

London kidney exchange in trouble

British transplant physicians are in hot water for allegedly transplanting kidneys obtained by purchase. But buying kidneys may not always be wrong.

In the design of mammals, there is a useful measure of redundancy: some organs, the kidney conspicuously, appear in pairs, the redundancy being a safeguard against the stresses of long life: if one of a person's kidneys should fail, the other may be sufficient. Kidney transplants are a further safeguard. Most transplanted kidneys derive from cadavers, but because a person is not fatally or even severely damaged by the removal of one kidney, some are from living people, usually close relatives of the recipient. Nobody disputes the value of the procedure (which in Britain is believed to save £50,000 for each renal patient taken off dialysis machines). Worldwide, some tens of thousands of transplant operations are carried out each year, mostly in developed countries. (Just under 1,700 cadaver kidneys were transplanted last year in Britain alone.) The success rate is high (about 80 per cent one year after the operation, half as much ten years later).

Physiological redundancy has now also, it seems, stimulated a trade in human kidneys. For the past two weeks, London newspapers have been in pursuit of what they allege to be an international racket. There is proof that Turkish newspapers carry advertisements from would-be donors. It is alleged that a two-legged conspiracy based in Istanbul and London arranges for donors to be shipped to London for operations and, afterwards, to be paid. Some of the subsequent transplant operations are said to be carried out at London hospitals in the private sector. That makes the issue especially piquant on the eve of the publication of the government's proposals for the reorganization of its National Health Service, known to include suggestions that major hospitals in the public sector may opt for autonomy, selling medical services to the public and private sectors alike.

Legislation

If (as is probable) there is a trade in kidneys from living donors, there will also soon be vigorous demands in Britain that it should be made illegal. As things are, physicians are enjoined by professional guidelines not to take part in operations in which organs from living donors have been sold, although close relatives of a person with kidney failure may donate a kidney out of altruism. There is also a Council of Europe declaration that would outlaw the practice of transplanting organs obtained by purchase from living donors — if the declaration had legal force. But the question of whether there are circumstances in which a person may be allowed to sell a kidney of his or her own for cash is more complicated than it may seem.

Operationally, the underlying principles are clear, even simple. Physicians with patients in need of a kidney transplant naturally owe their first duty to them, but are not thereby ethically absolved from the responsibility of enquiring into the means by which kidneys for transplant are obtained. In part to protect the zealous transplant physicians from their proper zeal, it is standard practice when transplant organs are obtained from patients who have died in intensive care that an independent physician is responsible for deciding when the putative source is brain-dead. The same principles should apply with the transfer of organs from one living person to another. It should be for a physician independent of the intended recipient to shoulder

ethical responsibility for the interests of the would-be donor.

Naturally, this second physician's chief responsibility (apart from seeking contra-indications to the proposed operation) must be to ensure that the intending donor is fully informed of the consequences, which are not as innocuous as the notion that one kidney is redundant would suggest. In normal people, kidney function becomes less efficient in middle life, and then progressively declines. However healthy a young donor may appear, losing a kidney must increase the risk of illness or even death in the second half of the normal lifespan. It is crucial to an understanding of what has been happening in London to know whether the Turkish donors so far tracked down were made fully aware of these extra risks. In principle, they might have been partly compensated by some form of health insurance, but there has so far been no suggestion of such arrangements. Indeed, the physicians most obviously concerned in the protection of the donors' interests have been singularly uncommunicative so far, yet they are those who shoulder the chief ethical responsibility.

With these provisos, there are two reasons why the practice of rewarding kidney donors even with sums of money large enough to provide an incentive should not be unthinkingly banned. First, there may be circumstances in which the balance of a potential donor's interests suggest that cash now justifies the risk of ill-health later. The simplest version of the hippocratic doctrine that no physician shall ever act so as to hazard a patient's health is easily challenged. What if the intending donor needs cash to care properly for a handicapped child, for example? Is the altruism that justifies the transfer of organs between relatives never transferrable to third parties? Even when there will be two beneficiaries (the recipient of the kidney and the object of the displaced altruism), not just one? It goes without saying that physicians will not win the freedom to use this or other possible shadings of hippocratic principles unless they are not merely willing, but eager, to describe and explain their conduct towards donors publicly — and to demonstrate that their own rewards are seemly. No doubt the medical profession's regulators will act with appropriate severity if these explanations do not emerge from the inquiries now under way in London.

The second reason why it is unwise to attempt to ban these practices is that the law is unlikely to be effective. Physicians where medical practice is relatively advanced inevitably attract patients from elsewhere. But the demand for kidney transplants is understandably so clamant that the trade (if there is one) will, if banned, shift to less squeamish places where standards of practice and (usually) ethics are high. Can that be to the greater good? Much the better way of blunting the problem is to ensure that the supply of organs for transplant is sufficient. In Britain, the National Transplantation Tissue Service, which operates within the public health service, is now able to meet only two-thirds of the demand for kidneys. British private-sector medicine derives a few dozen kidneys a year from dwindling unmatched supplies from the United States, but otherwise relies on living donors. Yet the shortfall is not so great, and might be made good by better international arrangements for collecting, matching and delivering kidneys to where people need them. That, not legislation, is the way out of this dilemma. □



Japan's science budget expands

- Spending reflects role as world power
- Massive budgets for space and nuclear power
- More money for superconductors and AIDS

Tokyo

JAPAN'S cabinet last week finally approved an expansionary ¥60.41 million million budget for fiscal year 1989. The budget is late, delayed by battles over the introduction of a new sales tax and by the resignation of three cabinet members who have admitted involvement in the Recruit stock scandal. But even with public confidence in the government at a new low, the economy is untroubled; a predicted 4 per cent growth rate lies behind the decision to let the budget rise 6.6 per cent over 1988.

Big increases in defence, overseas development assistance and student exchanges mark Japan's increasing awareness of its role as a world power. Science and technology budgets are also characterized by the appearance of new programmes to aid in the internationalization of Japan's research system, in line with the Council for Science and Technology's guidelines to emphasize international contributions and creative research and development in 1989.

All the international programmes are still tiny in financial terms, and despite the guidelines many had to be rescued at the very last minute from a hard-hearted Ministry of Finance. Even the Human Frontiers Science Program had to struggle for survival but it is finally under way, more than three years after first being

proposed, with a budget of ¥2,400 million (\$19 million) split between the Science and Technology Agency (STA) and the Ministry of International Trade and Industry (MITI). The programme is intended to promote basic biological research through international cooperation (see *Nature* **334**, 281; 1988) and will have its headquarters in Europe. London is the favoured location but Paris, Brussels or Heidelberg are still possibilities.

Both STA and MITI will also set up new organizations to look after foreigners coming to work at their research institutes. Problems of language, culture and accommodation have so far been left to each institute to solve (or abandon) in its own way. Increasing numbers of foreigners should be on their way to Japan, with the budget granting extra fellowships for scientists from the seven summit nations at STA and the Ministry of Education, Science and Culture (MESC). But filling those places already available remains a problem (see *Nature* **335**, 287; 22 September 1988).

Within STA, budget increases go to both the International Frontier Research System and Exploratory Research for Advanced Technology (ERATO) programmes. Both have been specially designed to break down the hierarchical structure of Japan's research laboratories

and to involve foreign researchers. But the really massive budgets go to space, where development of the H-II rocket is under way, to ocean research, where the Shinkai 6500 submersible has just been launched (see page xxx), and to nuclear power, where a special account provides large sums for studies of nuclear power plant safety. Opposition to nuclear energy is now increasing in Japan at a runaway pace. Earth science expenditure also booms with the launch of the Marine Observation satellite (MOS-1b) due for 1989, followed by the Earth Resources Satellite ERS-1 in 1991.

Expenditure at TRISTAN comes down (but running time goes up) as construction of the electron-positron collider is completed. Spending is also down on ocean sciences as the construction of the new oceanographic research vessel *Hakuo-maru* nears completion. Big increases are given to nuclear fusion research, to construct the world's biggest helical fusion device near Nagoya, and in space research to prepare for the 1990 launch of MUSES-1, a test satellite which will attempt to swing-by the Moon.

Both MITI and the Environment Agency join in international concern over global environmental problems, MITI with a initial 'concept' for a major project and the Environment Agency with a budget to develop an ozone monitoring device to fly on the Advanced Earth Observing Satellite 1993. The Environment Agency also receives funds to monitor acid rain and to put on showcase international environment conference in Tokyo.

International trends are also reflected in the predicted big budgets for research on superconductors, both high-temperature and conventional, at STA, MITI and MESC, and in increases for AIDS research by the Ministry of Health and Welfare (see *Nature* **335**, 194; 15 September 1988) even though the number of AIDS cases remains very small.

Both MITI and the Ministry of Transport are also looking at projects to tunnel beneath Tokyo at depths greater than 50 metres, beyond where landowners' rights end. Land prices are now so high that Tokyo could (in theory) be mortgaged for sufficient money to buy all the private land in the United States plus all the companies quoted on the US stock exchange (the transaction is not practical, although Japanese politicians like to joke about buying something smaller, California for example). The Ministry of Transport is interested in deep subways; MITI has a more futuristic plan to put factories in caves deep underground where even foreigners may be able to afford the rent.

Alun Anderson

Details of the Japanese science budget

	1989 ¥000 million	% change from 1988
Human Frontier Science Program	2.4	—
Science and Technology Agency		
Space	108.9	+10.6
Nuclear energy	279.9	+3.1
Ocean research	10.5	+11.1
Earth sciences	30.4	+72.0
ERATO	4.5	+18.4
International Frontiers	1.8	+11.8
Ministry of Education, Science and Technology		
Research grants	51.8	+6.6
Fellowships	1.9	+27.1
Accelerator physics	15.5	-6.1
Space science	21.0	+6.1
Nuclear fusion	9.2	+21.4
Marine science	2.9	-46.7
Ministry of International Trade and Industry		
New Energy and Industrial Technology Development Organization	7.2	+64.1
Basic technologies for future industries	6.8	+7.2
Large-scale industrial projects	14.0	+3.5
Sunshine project	37.5	+3.9
Moonlight project	10.5	+7.3
Fifth-generation computer	6.4	+12.5

UK universities request £183m

London

BRITISH university vice-chancellors last week asked the government for an extra £183 million over the next two years to help recruit and retain high quality staff and put an end to the pay dispute with academics, now into its fourth week.

The Committee of Vice-Chancellors and Principals (CVCP), asked for £64 million for 1989–90 and £119 million for 1990–91. This would finance a 6 per cent pay rise for all academic staff and leave some extra money to raise salaries in areas where there is a staff shortage and to reward staff of outstanding merit.

Academic salaries have fallen by more than 20 per cent relative to average earnings since 1979, said Richmond, in a letter to Kenneth Baker, the Secretary of State for Education. The CVCP has already offered staff a 3.5 per cent pay increase for this year but it recognizes that this is inadequate. If this offer cannot be improved, salaries will fall by a further 5–6 per cent in 1989, says Richmond, and to pay competitive salaries, numbers will have to be cut. The Association of University Teachers (AUT) has criticized the CVCP request, pointing out that even if this money is forthcoming, pay will still be 20 per cent behind average earnings relative to 1979 levels. It demands a substantial increase in basic pay levels, not just rewards to certain groups and individuals.

A survey for the CVCP has shown that 36 per cent of professorial posts and 19 per cent of lectureships were vacant last year. Recruitment of professors was particularly difficult in mathematics, accountancy, electrical engineering and computing. For lecturers, it was also difficult in general science, engineering and business studies.

The CVCP wants to add three discretionary salary points to the top of the lecturer pay scale to avoid having to promote to senior lecturer, which it says is unnecessarily expensive.

The committee also asked for £28 million for payment of overheads on research contracts. Because earnings from research councils and charities have increased, the University Grants Committee can no longer comply with its own instruction that universities should receive 40 per cent in overheads on research contracts, said Richmond. It also wants to increase technicians' salaries. In 1986, they were paid 18 per cent below market rates, and by April this shortfall will be 27 per cent.

The AUT boycott continues to disrupt examinations throughout the country; mid-session examinations are being cancelled and papers for summer examinations are not being set.

Christine McGourty

West German biochemistry institute set ablaze by "viruses"

Munich

MOLECULAR biologists in West Germany have been sleeping uneasily since 2 January, when arsonists broke into a laboratory in Darmstadt and started a fire that caused damage estimated at DM1.5 million.

Authorities say that the early-morning attack on the biochemistry institute of the Technische Hochschule in Darmstadt was the work of an 'autonomous' group thought to be loosely affiliated with the terrorist Red Army Faction. Because of the possible terrorist connection, the case is being investigated by the federal prosecutor-general. The only clue to the identity of the criminals is a letter, postmarked in Frankfurt, that was sent to news organizations.

In the letter, the "angry viruses" (*zornige Viren*) — a previously unknown group — take responsibility for the attack. The letter, written in a convoluted style, calls genetic engineering a tool of Western imperialism used to exploit the developing world. The use of such techniques in agriculture, says the letter, is directly related to the "programmed starvation" of subsistence farmers. Human genetics and reproductive technologies are the "definitive attempt" to subject the fertility of women to masculine exploitation and control.

The director of the biochemistry institute, H. G. Gassen, is singled out in the letter as a "clever propagandist of genetic engineering". Gassen had become known

locally as the initiator of a university-industry consortium for pharmaceutical research. The letter accuses Gassen of trying to increase public acceptance of genetic engineering by acknowledging specific criticisms but defending the research in general.

The attack is the latest in a series of assaults on research institutes and private companies that use molecular biology techniques. Earlier attacks have been reported in West Berlin, Cologne, Braunschweig, Münster, Hannover and Heidelberg.

The West German press and the public have expressed strong reservations about genetic engineering in recent years. West German police have alerted other private and public research institutes of the danger of a possible repeat attack.

Gassen, whose work involves the development of synthetic enzymes for diagnostic purposes, says he will not let himself be intimidated by the attackers. "That's just what they want — for us to perform our research in secret behind locked doors. We refuse to play their game." The fires were set in four places on the sixth floor of the biochemistry institute using bags of gasoline hooked to a welding kit and a timing device. Gassen's isotope laboratory was left coated with soot and will be unusable for at least three months.

The fire would have endangered "ten years of research", says Gassen, but it was extinguished by the fire department before it could do more damage.

Steven Dickman

Seasonal greetings at high dilution



THE Department of Pharmacology at the Free University of Amsterdam follows the practice of commissioning a solstitial greetings card from its two newest members. This year's card is reproduced above. □

New warmth via research scheme

Tokyo

THE first meeting last month of the Joint High-Level Advisory Panel set up under the new US-Japan Science and Technology Agreement focused on problems of 'comparable access to common base technology' in Tokyo. But Japanese representatives also expressed interest in participation in research on global environmental change, a new area for Japan.

According to officials at Japan's Foreign Ministry, Prime Minister Noboru Takeshita has been advised that Japanese participation in research on the greenhouse effect and acid rain would be well received in the United States. Takeshita intends to host a conference on global environmental problems in Tokyo this year and may propose a joint US-Japan research programme when he visits Washington next month.

The fourteen members of the advisory panel include Deborah Wince-Smith, assistant director of the US Office of Science and Technology Policy, Mary Good, chair of the US National Science Board, and Robert White, president of the US National Academy of Engineering. Among the Japanese members are Michio Okamoto, former president of Kyoto University, Saburo Nagakura, president of the Graduate University of Advanced Studies, and Minoru Oda, president of the Institute of Physical and Chemical Research. Wince-Smith said that discussions had been "free" and "very positive" and that the group did not try to "revisit" the issues that made negotiation of the US-Japan agreement long and difficult. But several Japanese participants noted that the problem of "comparable access" was not easy to solve quickly.

Both nations would like to see equal access to each other's research at the 'generic' or 'pre-competitive' stage, that comes before 'applied research' generates actual products. The problem is that whereas US base technology is often developed in government laboratories, to which foreign researchers have access, much Japanese technology is developed in private companies closed to outsiders. The US government has considerable leverage even over research performed in private laboratories, as much of it is paid for by the Department of Defense funds. The same is not true in Japan.

Japanese panel members stressed that progress towards putting foreign researchers in company laboratories is likely to be slow, and that much discussion is needed. Some members believe the quickest way to increase joint access to basic research would be to strengthen Japan's university system. **Alun Anderson**

Foreign critics hit a nerve in self-conscious West Germany

Munich

FOREIGN researchers' eloquent criticisms of academic life and society have caused a stir in West Germany, a country unusually concerned about what foreigners think of it. The criticisms appear to have hit a raw nerve throughout the country, provoking a response of shame and self-analysis. West Germany has had to cope with a rising number of foreign workers and ethnic German 'returnees' from Eastern Europe.

The widely reported comments appear in a report (*Through the eyes of others: experiences of foreign researchers in Germany*) issued by the respected Alexander von Humboldt Foundation. The non-profit foundation brings hundreds of researchers to West Germany every year for periods up to two years. It has supported more than 12,000 foreign researchers in the past 35 years.

The negative experiences described in the report stand in sharp contrast to the generally glowing reviews received in the past. This may in part be explained by the fact that Humboldt fellows have only recently been required to fill out questionnaires about their stay in Germany. Voluntary contributions formed the basis of earlier reports issued in 1965 and in 1977.

Although the overwhelming majority of researchers felt they profited from their stay in West Germany, the criticisms attracted the most attention. Most disturbing to West Germans was the increasing xenophobia and insensitivity that the researchers encountered in West German laboratories, libraries and on the streets.

One frequently quoted account was by a black South African scholar who first came to West Germany in 1958. He wrote that in his first three-year visit he had been openly confronted with racism only once and had struck up friendships with West Germans on the street and in the labour unions where he sometimes worked, leading him to consider West Germany his "second homeland". Twenty years later, he felt "wounded" by "harsh, unpremeditated words flung at him" in the streets.

Although his experiences are more extreme than many of those described by foreign researchers, the image of "material riches" accompanied by "emotional poverty" was a common theme in their reports.

In addition to its message for West German society at large, the report included criticisms of everyday university life. Comments such as "more confrontation than cooperation" and criticisms of the hierarchical organization were commonplace, especially from West European or North American researchers. One researcher wrote that "German professors are the last German kings".

Despite the criticisms, Heinrich Pfeiffer, general secretary of the Humboldt Foundation, said that in the past eight years the foundation has been "overrun with applications" from all over the world. It is at present planning a drive to raise money so that it can offer a hundred new fellowships to researchers from China and the Soviet Union. There have been growing numbers of applications to the foundation from both countries in recent years.

Steven Dickman

Harvard group to teach Soviet diplomats

Boston

THE Soviet Ministry of Foreign Affairs has announced that it will accept an offer by the Harvard Negotiation Project to conduct a five-day 'negotiation workshop' for mid-career Soviet diplomats in Moscow this spring.

Although bilateral cooperative efforts have lately been flourishing between the United States and the Soviet Union, negotiation skills of diplomats have not kept pace with the improved climate, according to the Negotiation Project's director Roger Fisher.

Fisher, a professor at Harvard Law School, says the new arrangement represents "a crucial, early step in the development of a needed skill", namely to learn "how to handle differences" between the two nations through diplomatic channels.

The negotiation workshop will be held at the Soviet Ministry's Diplomatic Academy in Moscow, which provides advanced training for experienced Soviet diplomats.

Among Fisher's suggestions for alternative processes are "pre-negotiations" where parties sit down together to reach a mutual understanding of the scope and quality of the outcome desired through the negotiation process. Fisher calls international conflict "a growth industry", and says that improving conflict resolution procedures is necessary "in order to make the civilized world work". Now that the Soviets have agreed to a workshop, the Negotiation Project wants to persuade the State Department of the United States to accept a similar arrangement. So far, the State Department has not accepted the offer.

Seth Shulman

European molecular biology laboratory in search of a director

London

THE withdrawal of the only candidate for one of Europe's top scientific posts has created a considerable problem for the governing body of the European Molecular Biology Laboratory (EMBL) in Heidelberg. The most likely outcome of a meeting of EMBL's council on 6 February is that the present director, Lennart Philipson, will be asked to stay on beyond April 1990, when he was to have been replaced.

The hunt for a successor to Philipson, who has been director since 1982, has been under way for two years. A search committee chaired by the council's chairman, Professor Jan Drenth, scoured Europe for suitable candidates and identified a handful. But only one of them, Professor Tom Blundell of the Crystallography Department, Birkbeck College, London was interested. He withdrew his candidature two weeks ago after some months of negotiation.

At a council meeting last December, it became clear that a small number of the 14 countries that provide EMBL's funds were opposed to Blundell's appointment, and a decision was postponed until next week's special meeting. There has been little or no weakening of the opposition and it seems unlikely that the ten votes

necessary for the appointment would have been cast at the meeting.

One reason given for opposing the appointment, particularly by the French delegation, was that EMBL should not have a structural biologist as its director. But there was also opposition, much of it from within EMBL, to Blundell's condition that he should be allowed to continue his personal research, even though his proposal was for a modest group of 10 people.

Blundell says he had been greatly looking forward to the challenge of the job, and particularly the task of sorting out the central facilities of EMBL, including its out-stations and the biocomputing laboratory, whose work falls within his own expertise. Instead, he now hopes for rapid progress in his negotiations with the Imperial Cancer Research Fund for a substantial programme of collaborative research.

EMBL's council is faced with the choice of restarting its search for a successor to Philipson or extending his stay. Drenth feels that it would now be in the best interests of the laboratory if Philipson would stay on. And Philipson is believed to be willing to stay if certain conditions are met.

Peter Newmark

BMA scorns loans

London

THE British Medical Association (BMA) this week criticized the government's plans to introduce a loans system for students to supplement the current grants system, saying that students would be deterred from entering the medical profession, especially those from poorer families. The profession is disturbed at the prospect that its future stock will be drawn from those who can afford to pay for a medical education rather than those best suited by their ability to benefit from it, says the association, in a letter to the Department of Education and Science.

Surveys reveal considerable disillusionment among young doctors about careers in medicine, says the BMA. Of those qualifying in 1981, 46 per cent expressed regret at having entered medicine, compared to 28 per cent in 1976. If young doctors are also faced with large outstanding loans from their undergraduate days, this disillusionment will only get worse. The association estimates that by 2006 a junior doctor could have a total loan to repay of up to 35 per cent of his or her first year's income.

The BMA urges the government to reconsider its plans for a loans scheme and instead to restore the recent loss in real value of the grant. Christine McGourty

Arianespace order

Paris & Tokyo

THE night of 27 January was a busy one on launch pads around the world. At 01.21 GMT, Arianespace, the consortium operating European space launches, put an Intelsat V communications satellite into geostationary orbit. Just a few hours earlier, on the other side of the globe, the Japanese National Space Development Agency successfully launched the TR-1(2) prototype for its H-II rocket.

The European launch from Kourou in French Guiana confirmed Arianespace's lead in the commercial satellite launch market. With an order book for 36 launches, nine of them scheduled for this year, Arianespace hopes to repeat last year's turnover of \$520 million.

In February, Arianespace will sign an order for 50 Ariane-4 launchers, worth more than \$4,500 million, and intended to be the company's workhorses until 1999. Japan hopes one day to take a slice of that market. But it will be at least 1992 before the H-II rocket is complete, and commercial launches are not expected to begin until the late 1990s. By then, H-II's 4-tonne payload may look small compared with that of planned new Ariane launchers.

Peter Coles & Alun Anderson

New chairman

Munich

THE West German Wissenschaftsrat (science council) chose Dieter Simon as its new chairman at a meeting in West Berlin on 27 January. Simon, a law scholar and director of the Max Planck Institute for European legal history in Frankfurt, replaces physician Kurt Kochsieck of Würzburg.

Simon led a strike of the scientific review commission of the Wissenschaftsrat in December of last year (see *Nature* 336, 506; 8 December 1988) to protest at the demotion of the council's general-secretary to the lowly status of a public servant. Pressure from the political heads of the *Länder* in late December persuaded the federal government to restore the general-secretary position, currently unoccupied, to its original higher status.

In his final statement, Kochsieck pleaded with the *Länder* to increase their education budgets by twice as much as they had planned in order to alleviate overcrowding and a decline of quality at West German universities.

S. D.

New NSF positions

Washington

RICHARD Nicholson, at present assistant director for mathematical and physical sciences at the National Science Foundation, was last week named as the new executive officer of the American Association for the Advancement of Science. He replaces Alvin Trivelpiece who left the association at the end of last year to become director of the Oak Ridge National Laboratory. Nicholson is expected to join the association by mid-April.

The foundation also announced last week that Mary Clutter would take over as assistant director for biological, behavioural and social sciences. She replaces David Kingsbury, who resigned last October amid allegations of conflict of interest over his dealings with a biotechnology company.

J. P.

Accelerator all set

Washington

BACKERS of the Superconducting Super Collider, the proposed \$4,400-million proton accelerator, now have everything they need except congressional approval for construction funds. Two weeks ago, Waxahachie, Texas, was confirmed by outgoing Secretary of Energy John Herrington as the site of the Superconducting Super Collider after a review of the full environmental impact statement, and last week the Department of Energy signed a nine-year contract with the Universities Research Association, a consortium of 66 universities, to manage and operate the facility. The association runs the Fermi National Accelerator Laboratory near Chicago, and was the only bidder.

D.L.

Rifkin tries to block human gene transfer experiment

Bethesda

THE first experiment to insert foreign genes into humans, which received approval to proceed only two weeks ago, may be blocked by Jeremy Rifkin, an active opponent of genetic engineering.

On 30 January, Rifkin's Foundation on Economic Trends filed a suit in a US federal district court to stop W. French Anderson of the National Heart Lung and Blood Institute and R. Michael Blaese and Steven A. Rosenberg of the National Cancer Institute from carrying out the experiment. The plan is that tumour-infiltrating lymphocyte (TIL) cells marked with a gene for antibiotic resistance will be injected into ten cancer patients from whom the cells were isolated.

Rifkin has filed his suit because he says the approval process of the National Institutes of Health (NIH) did not fully review the experiment or allow for public debate in the final stage. But NIH officials point out that there has been ample opportunity for open discussion and the experimental protocol has undergone a complex and lengthy review process.

The road to approval for the gene transfer experiment has not been smooth. Anderson and his colleagues first proposed their experiment last June, and it was referred first to various institutional review committees and then to the NIH Recombinant DNA Advisory Committee (RAC) for approval. A RAC subcommittee on human gene therapy first concluded that more data were needed, but at the end of September, Anderson told the subcommittee that he did not have the data it was seeking, whereupon the committee deferred approval of the protocol.

But on 3 October, Anderson presented much of the same data to a full meeting of the RAC, which approved his experiment. NIH director James Wyngaarden ordered that the subcommittee be given a second chance to review the proposal in the light of the new data, and the application was afterwards resubmitted to the full RAC, which approved it by mail ballot.

Rifkin objects to the mail ballot. Andrew Kimbrell, policy director and attorney for Rifkin's foundation, says that after the 3 October meeting, significant changes were made to the experimental protocol which under federal law should have been debated in an open RAC meeting, adding that the RAC review process in this case has been "confused, backwards and illegal". Speaking at this week's RAC meeting, Wyngaarden countered that there were no substantive changes to the protocol approved at October's open RAC meeting, so a further public meeting was unnecessary. The gene therapy com-

mittee's final deliberations, held on 9 December, were public.

But Wyngaarden does agree that the Anderson case has pointed to a problem with RAC procedures. He announced last Monday that he would seek an amendment to the RAC charter requiring that protocols referred to a RAC subcommittee be approved by that subcommittee before being put to the full RAC.

Anderson says he thinks Rifkin's lawsuit is "a good thing" because it will bring the issue out in the open for public discussion. But Rosenberg points out that an American dies from cancer every minute, and "any delay for any reason would be

very unfortunate."

Rifkin also used the forum of the RAC meeting to propose an amendment to NIH guidelines that would establish a Human Eugenics Advisory Committee to provide advice on the "ethical, philosophical, social, economic and eugenic implications and impacts of human genetic therapy".

But RAC chairman Gerard McGarrity pointed out that the RAC does not have the administrative authority to create such a committee. Such amendments can only be made by the Secretary of the Department of Health and Human Services who approves RAC's charter. Nevertheless, the RAC spent nearly two hours debating the merits of the proposal, and although there was general support for the idea, most felt existing review bodies could cope with the issues that a eugenics committee would address. **David Swinbanks**

Errors in US census rouse political protests

San Francisco

TIME is running out to resolve the political and legal challenges to the way the 1990 US census will be conducted. Yet practical issues such as how to count the minority population of large urban areas and whether to count illegal aliens will have a profound effect on the social fabric of the United States in the next decade.

The decennial census is used not only for allocation of congressional representatives among the 50 states, but also for the distribution of federal funds to states and cities for services such as hospitals, schools, highways and housing. But the census usually misses 10 per cent or more of the minority population in some urban areas, and the cities most hurt by the undercount have accused the Bureau of the Census of ignoring a proven statistical means of correcting the numbers.

Census bureau decisions are subject to strong political pressures, as a small change in population can mean the gain or loss of a congressional seat. Since 1970, the census has been taken largely by mailed questionnaires; enumerators are sent in person only to households that do not respond. The method runs into difficulties in poor urban neighbourhoods.

After the 1980 census, the census bureau faced 37 lawsuits by areas contending that their minority population had been undercounted, all of which were unsuccessful. Nevertheless, the bureau developed a method called the Post Enumeration Survey (PES), using an in-depth survey of 300,000 households drawn from representative city blocks, to adjust its first counts. A test of PES in Los Angeles demonstrated a 9 per cent undercount in black neighbourhoods.

Barbara Bailer, chief of the team that

devised the method, acknowledges that PES will still miss people who actively avoid being counted, but says it will nevertheless bring the numbers closer to the truth. Despite endorsements from three scientific committees, the census bureau has decided not to use PES to adjust the 1990 census. The bureau claims its existing methods are sufficiently accurate, and that PES would be too expensive.

Bailer resigned in protest at the decision, complaining that the census uses other, less-sound adjustment methods costing more than the \$31 million required for PES. Some claim that the decision is politically motivated by Republican fears of losing congressional seats, as the undercounted populations typically vote Democratic.

Meanwhile, a coalition of cities and minority advocacy groups has launched a lawsuit to compel the bureau to shift to PES. A bill with the same objective introduced in Congress was killed last September by the addition of an amendment requiring the exclusion of illegal aliens from the census. The census does not at present aim to determine citizenship, and the census bureau is being sued by a coalition of congressmen who oppose counting illegal aliens for apportionment of congressional seats.

The census bureau defends the inclusion of illegal aliens on the grounds that the constitution requires counting all residents. In any case, says Jeffrey Passel of the census bureau, excluding illegal immigrants would be difficult because it would require the determination of citizenship, requiring all non-citizens to be matched against Immigration and Naturalization Service records, known to be in disarray. **Marcia Barinaga**

Hungary breaks science links with Romania over Transylvania

London

A LETTER from Romanian scientists appealing to Hungarian colleagues not to break off scientific relations was recently read at a closed session of the Hungarian Academy of Sciences. This emerged at a meeting of the Hungarian government's cultural and foreign affairs committee last month. But the letter came too late. Talks between the two governments on cultural, educational and scientific cooperation seem to have broken down over the issue of Transylvania.

The dispute between the two countries dates back to the settlements after the First World War, when the Treaty of Trianon awarded Transylvania to Romania. But the Hungarian presence in Transylvania goes back more than a thousand years — Magyars were there before occupying what is now Hungary in 895 AD. In the sixteenth and seventeenth centuries, Transylvania was for a time the heartland of Hungarian resistance to the Ottoman Empire.

The Romanian policy of de-Magyarization of Transylvania began in 1918. Since the Second World War, despite the establishment of socialist regimes in both Hungary and Romania, Romanian policies in Transylvania have continued, but Hungarians have been reluctant to criticize the Romanians in public.

The Hungarian "Bolyai" University at Cluj-Napoca (formerly Kolozsvár) was closed down (the Rector committed suicide) and, during the 1970s and 1980s, reports of discrimination against Transylvanian Hungarians in education and professional employment became increasingly frequent and alarming.

Since 1987, the Romanian government has been "consolidating" agricultural settlements, which means the destruction of the villages of Transylvania and the resettlement of the inhabitants in high-rise "agro-industrial settlements". Thousands of Transylvanian Hungarians have fled across the border, swamping the Hungarian social services. Private initiatives and action by the churches have, at best, offered only temporary relief. There are 2.3 million Hungarians in Transylvania but only 10.5 million in Hungary itself.

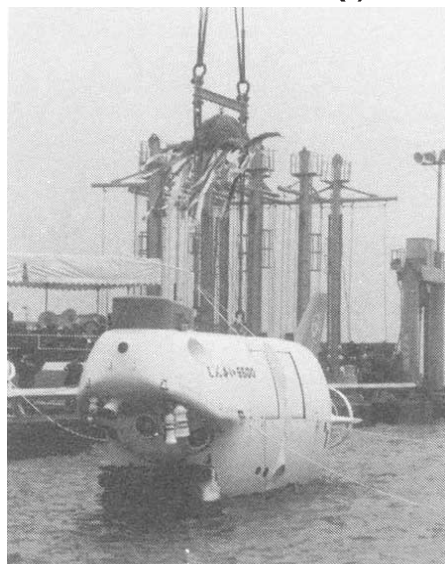
Diplomacy by the Hungarian government has come to naught. The Romanians insist that the destruction of the villages is "rational" and "scientific" and point out that they have sent in "conservation" officers to photograph anything of interest before it is bulldozed.

Increasingly, the Hungarians are calling for an "international" solution. In an unprecedented move for a Socialist country, Hungary has invited a delegation from

the UN High Commission for Refugees, which is due in Hungary next week. There have also been appeals by Hungarian "democratic" and "informal" movements in Hungary for the application of sanctions to Romania.

In Britain, there have been several calls for Madame Elena Ceaucescu to be stripped of the academic honours conferred on her by such bodies as the Royal Society of Chemistry. Although Ceaucescu is not, formally, responsible for the destruction of the villages, she is widely believed to be the real power behind the president. Although she is acclaimed as Romania's greatest scientist, others have questioned the quality and originality of her publications. But the Royal Society of Chemistry says that Ceaucescu was elected as a Fellow to the Royal Institute of Chemistry in the regular manner on the basis of her chemical career and publications, and that the charter of the Royal Society of Chemistry provides for the expulsion or suspension of a fellow only in the case of a criminal conviction, failure to observe the Society's bylaws or "conduct detrimental to the welfare of the society". **Vera Rich**

Sub's first outing



SHINKAI 6500, designed to be the world's deepest diving research submarine, took its first dip at Mitsubishi Heavy Industries shipyard in Kobe, Japan, last week. Its titanium alloy hull and pair of external manipulators will allow it to collect samples from the ocean bottom at depths of 6,500 metres, 500 metres deeper than vessels operated by other countries. After sea trials, the submarine will be delivered to the Japan Marine Science and Technology Center (JAMSTEC) in November. JAMSTEC will use the submarine to investigate the causes of undersea earthquakes, to assess deep ocean-bottom mineral resources and to study deep-sea life.

Alun Anderson

New complaints about US waste in the Antarctic

Sydney

GREENPEACE in Australia has joined environmental groups in the United States in accusing the US government of leaving behind an environmental mess at research facilities in Antarctica. A scientific committee on Antarctic research, made up of scientists from Antarctic Treaty signatory countries, will meet next October to recommend new waste disposal practices for the Antarctic.

The Australian group is focusing on two bases in the Antarctic — Wilkes, a former US and Australian base, and McMurdo, the present US base. Wilkes was established by the United States in 1957 as a temporary base before McMurdo was completed. In 1964, the Australians took over the abandoned base, where they remained until 1969 when they moved to their present base, Casey.

According to a spokesperson for Greenpeace Australia, Lyn Goldsworthy, when Wilkes was abandoned "a lot of rubbish was just left behind". This included 200–300 petrol drums, many still full, old food sacks and derelict buildings.

In 1985, the US government gave Australia permission to clean up Wilkes. Rex Moncur, acting director of the Australian Antarctic Division, says that although the Wilkes buildings are technically US property, Australia accepts responsibility for cleaning up the site, and plans to start work at the end of this year.

Greenpeace in Australia claims that McMurdo is still generating a dangerous amount of waste. Goldsworthy says McMurdo's sewage system discharges directly into the sea, and that some parts of the ocean floor off McMurdo are heavily contaminated with polychlorinated biphenyls. She says Greenpeace has evidence that waste items such as batteries have been found at dump sites, contravening the code of conduct of waste disposal of the Antarctic Treaty.

Jack Talmadge, section head for polar coordination and information at the United States National Science Foundation, says the US government has always taken the waste problem seriously, and the recent reports have merely focused outside attention on the issue. Talmadge says the foundation's budget request for 1990 includes initial money for a four-year, \$30-million safety and environmental health initiative that will clean up existing problems and establish new procedures and equipment to forestall new ones. "We're taking the high road in getting the job done", he says.

Tania Ewing

Science literacy found wanting in United States and Britain

San Francisco

MORE than 90 per cent of adults in the United States and Britain are scientific illiterates, according to survey results released last month at the annual meeting of the American Association for the Advancement of Science in San Francisco. Its authors claim that it is the first such survey to be carried out in both countries, allowing comparisons to be made easily.

The dismal state of scientific literacy in the United States has recently prompted renewed attention to science education. The new surveys, developed in the United States, were designed to determine whether people have a minimal level of scientific literacy. To test for a basic understanding of scientific methods, respondents were asked "what does it mean to study something scientifically?".

Answers judged correct included testing of hypothesis and building of theory, the use of experiments, or systematic

comparative study. As a check on this understanding, those who answered the question correctly were then asked to say whether astrology is "very scientific", "sort of scientific" or "not scientific at all". Only those who said astrology is not scientific at all were classified as having a general understanding of how science is conducted.

Simple questions such as "does the Earth go round the Sun, or does the Sun go round the Earth?" were used to judge understanding of scientific concepts. To assess understanding of the impact of science on society, respondents were asked questions such as "is it true radioactive milk can be made safe by boiling it?". (After the Chernobyl accident, a household in Cumbria in northern England was found to be boiling milk as a protective measure.)

Only six per cent of US adults and seven per cent of British were found to have at least minimal understanding of the process, concepts and impact of science. The US results were comparable to those of earlier surveys in 1979 and 1985.

Jon Miller, director of the Northern Illinois University Public Opinion Laboratory, who carried out the US surveys, blames the widespread scientific illiteracy on inadequate education, particularly at the high school level.

But some question the validity of the surveys, claiming that they tend to overestimate illiteracy because of misunder-

standings of the questions. As an example, some members of the general public confuse astrology with astronomy.

There are interesting differences between Britain and the United States in questions relating to medicine. In a self-assessment, more US than British respondents (80 per cent against 50 per cent) said they are "very interested" in new medical discoveries. Similarly, about 70 per cent of US adults consider themselves "moderately" to "well informed" about such discoveries. But the high self-assessment does not translate into knowledge.

On several medical questions, US adults actually fared slightly worse than their UK counterparts. For example, 75 per cent of US respondents falsely believe that antibiotics are effective against viral infection, as against about 70 per cent of British. And although US adults were better at identifying risk factors for heart disease with more than 90 per cent correctly pinpointing smoking, lack of exercise, stress and eating too much animal fat, higher percentages of US adults (50–65 per cent) falsely believe that food additives and lack of vitamins, fresh fruit and fibre contribute to heart disease.

Miller says the dismal results in both countries are a "threat to democracy". He notes that science and technology are playing an increasing role in society but that the vast majority of people are not sufficiently literate in science to make informed decisions about major issues related to science.

Miller's solution is a radical improvement of the scope and quality of pre-university science education.

David Swinbanks

RVS move opposed

London

THE plan of the UK Natural Environment Research Council (NERC) to create a centre of excellence in oceanography at the University of Southampton will have to be redrawn to exclude the transfer of the Research Vessel Services (RVS) from Barry in South Wales, if staff at the base get their way.

The RVS provides the shipborne instrumentation, data acquisition and computer facilities necessary for marine research programmes of NERC and universities throughout the country. More than 60 of the 70 members of the RVS staff based at Barry are opposed to the move, according to one member of a committee formed to campaign against the relocation, who asked not to be named as staff have been asked not to speak to the press.

The committee has written to council members of NERC and to the secretaries of state for science and for Wales protesting against the plan, arguing that groups throughout the country rely on the services of RVS and that relocation at Southampton cannot be justified on the grounds of improved accessibility to the unit's services. They say that the move will cost £10 million, a sum that could be better spent on modernizing the equipment of the RVS. Creation of the centre at Southampton will cost £35 million over five years. The plan will go ahead if the government agrees to provide the money; an announcement is expected in the next few weeks.

Christine McGourty

Protection of endangered species threatened

Washington

PROGRAMMES to safeguard endangered species in the United States are themselves threatened by a lack of funds, says a report released at the end of January by the Government Accounting Office (GAO).

The Endangered Species Act of 1973 requires that the responsible agencies, either the Fish and Wildlife Service (FWS) of the Department of the Interior or the National Marine Fisheries Service (NMFS) of the Department of Commerce, should develop and put into action recovery programmes for species classified under the act. Although the GAO makes no attempt to assess the biological efficacy of these programmes, it finds that for 40 per cent of the classified species no recovery plan has been formulated, and that in the 18 cases it reviewed in detail only half of the steps in the plans have been initiated. The problem is compounded by failure of the FWS and NMFS to follow systematically the progress of its recovery plans.

Of the 482 species classified as threat-

ened or endangered, fewer than one-sixth, according to the report, are increasing in numbers, and one-third are continuing to decline. The report also identifies a failure within FWS, which is responsible for 464 of the 482 classified species, to assign priorities to various species according to its own guidelines. This is partly due to pressure from Congress, which in 1987 forced FWS to spend 11 per cent of its recovery funds on only eight species, and partly due to a perceived need by FWS to provide a strong effort in some highly visible cases, such as the American bald eagle.

The GAO lays most of the blame on a simple lack of money, as well as organizational deficiencies in FWS and NMFS. But it also recognizes that recovery of some species may be unlikely, regardless of human efforts: the fragile desert habitat of the New Mexico ridgenose rattlesnake and the Texas beaches on which Kemp's Ridley sea turtle breeds are steadily shrinking, despite conservation efforts.

David Lindley

"Erosion of UK research on agriculture and food must end"

London

Cuts in government support for near-market research were severely criticised last week by the House of Lords Select Committee on Science and Technology. In a report on agriculture and food research in Britain, it said the government should reassess the list of projects defined as near-market so as not to include research which is in the public good, such as animal health and welfare, food safety and quality standards and protection of the environment.

The committee also recommends that the proposed timescale of three years for the transfer to industry of support for near-market research should be extended to five. Savings made in this exercise should be used to support basic and strategic research.

Although some of the rationalization in the Agricultural and Food Research Council (AFRC) has been to good effect, the cuts and the uncertainty about future

towards more short-term appointments. Such appointments are appropriate when projects are of fixed length, but should not be used simply because future finances are uncertain. In the AFRC, the number of short-term appointments is already too high, it says.

The Ministry of Agriculture, Fisheries and Food comes under searing criticism from the committee. Scientific knowledge has had little impact on the ministry's policy, it says; because of the inadequate number of staff and an inappropriate structure, interaction with researchers is inadequate, as is the provision of proper guidance and advice.

The committee also criticizes the ministry's performance as a customer for research. Although it supports the principle that applied research should be supported on a customer contractor basis, its performance has been poor. Because of

this, the committee recommends that funds transferred in 1975-76 to the ministry from the then Agriculture Research Council should be returned to the AFRC, or given to the proposed Natural Resources Research Council (NRRC). (In its interim report on agriculture and food, the committee recommended that a new council, the NRRC, be formed from a merger of the AFRC with the Natural Environmental Research Council.) The money — about £27 million — would be more productive as part of the AFRC or NRRC budget, says the committee.

The Biotechnology Directorate of the Science and Engineering Research Council is praised by the committee, but it says that the other research councils should be involved in the directorate's activities. There should be more joint initiatives, for which proposals should come from a new advisory group. It says that more research is needed on plant biochemistry and physiology, and it recommends more research on nutrition and forestry.

Christine McGourty

US plan for proton accelerator to produce tritium for warheads

Washington

AN old idea for producing tritium for nuclear warheads is gaining new favour in the United States. A report compiled by scientists and engineers at Los Alamos National Laboratory, Brookhaven National Laboratory and Westinghouse Hanford Company, which is soon to be submitted to the Department of Energy (DoE), advocates construction of a huge linear proton accelerator for the production of tritium; it may be powered by excess electricity from hydroelectric sources.

The closure last spring of the ageing Savannah River reactors, the sole US source of tritium for nuclear bombs, forced DoE to consider alternative production methods. Last summer, the department announced plans for two new production reactors, a heavy-water reactor at Savannah River and a modular high-temperature gas reactor at the Idaho National Engineering Laboratory (see *Nature* 334, 558; 18 August 1988). The DoE budget request for 1990, compiled by the former Reagan administration, includes some money to start building the reactors. But a draft of the Los Alamos-Brookhaven-Westinghouse report claims that accelerator production of tritium would be cheaper and safer.

The principle of accelerator production is simple. A beam of high-energy protons is fired at a lead target to produce high-energy neutrons which in turn hit a lithium target which emits helium and tritium.

The same approach is used in production reactors where the source of the neutrons is nuclear fission.

Wolfgang Panofsky, emeritus director of the Stanford Linear Accelerator Center, says the proposed accelerator is "well within the capabilities of present technology". In fact, he says, a prototype accelerator with a proton beam current comparable to that proposed (250 milliamps) was built at Livermore for the production of plutonium and tritium in 1949. Panofsky also agrees with the report's conclusion that the accelerator system will produce less radioactive waste than a production reactor.

But a key problem will be providing the gigawatt of electricity the accelerator will need. Building a new nuclear reactor to supply the accelerator would defeat the original purpose. But Pierre Grand of Brookhaven, who helped to compile the report, says that Bonneville Power Administration in Washington state, where advocates hope to site the accelerator, has tentatively said it can provide about 900 MW from excess hydroelectric generating capacity.

Another open question is cost. The report estimates that the 1.6-GeV facility will cost about \$2,300 million with annual running expenses of \$250 million — considerably cheaper than the estimated \$6,800 million required for new production reactors. But Grand emphasizes that these figures are "very preliminary".

David Swinbanks



levels of support have caused instability and severe problems in the research community, says the committee. Industry should make a major contribution to the cost of research aimed at increasing industrial profitability and competitiveness, but the committee warns that if a sound research base is not maintained through public support, then commissions are likely to go overseas.

The research base in agriculture and food should not be reduced further, says the committee. A period of stability would lead to a reversal of the damaging trend

Genetics of schizophrenia

SIR—As is his wont, Steven Rose (*Nature* **336**, 512; 1988) belittles the efforts of molecular biologists to understand human problems. Rose's objection that "Sherington *et al.* have not shown that [the locus on chromosome 5 to which their RFLP markers are linked] is sufficient [for a person to develop schizophrenia] — a prerequisite for useful genetic counselling — is misleading on two points. First, Sherington *et al.* (*Nature* **336**, 164–167; 1988) did not claim that they had found a locus which inevitably leads to schizophrenia. They specifically quote a penetrance of 85 per cent even given a broad definition of schizophrenia-related diseases, stating that there are influences at work other than the genetic ones they have identified. Second, the locus is useful for genetic counselling whether or not it determines schizophrenia in the way that Rose would require of it. Genetic counselling has for many years relied on statistical arguments, often stemming only from mendelian genetics, with no linked markers at all. Many couples have felt that to be told that their children have a 25 per cent change of suffering from (say) cystic fibrosis is better than knowing nothing. Even with the advent of more statistically certain markers, such as the G8 probe which is linked to the locus causing Huntington's chorea, it is clearly recognized that possession of the locus is quite compatible with survival to 70 years of age with intact mental faculties. As always, genetic counselling is not only genetics — it is also counselling on the interpretation of those genetics.

This is exactly the reverse of the reductionism that Rose so vividly describes, but which is not actually present in your original articles: it is placing human interpretation on molecular results. That Rose invokes this argument only to knock it down as counter to a human science is at least consistent with itself, if not with the

facts. That he then uses it to argue against attempts to develop more carefully targeted drugs to treat incapacitating mental disease is obtuse. Of course this course has not been successful in the past. It has not been available in the past. On this argument we would still be treating schizophrenia with the ducking chair and the stake.

WILLIAM BAINS

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Reprints justified

SIR—I agree with Ivor Smith (*Nature* **336**, 708; 1988) that sending reprints is costly and time-consuming. In places such as Italy however, photocopiers abound, but university libraries do not subscribe to major journals, or journals are distributed only after months of delay. In such cases, it is vital for investigators to receive reprints (or even cheap photocopies) of papers within a few weeks of their listing in *Current Contents* or similar indexes.

MARCO RUGGIERO

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SIR—I request reprints because it saves time. I subscribe to *Current Contents* and, on average, request three or four reprints per week, which takes about half an hour. If I were to go to the library, find the journals and copy the articles, it would probably take at least half a day. And often the journals that I need are not in the library because of budget cuts.

I feel that an investigator has the obligation not only to do research but also to disseminate it. Most administrators would probably be glad to provide the cost of photocopying and postage to have research at their institution known and cited.

M.B. KIRKHAM

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Swiss academic strife

SIR—The picture drawn by John Maddox (*Nature* **336**, 333; 1988) of Swiss academic life is in my opinion a bit too rosy. I agree that there are a few positive aspects: faculty salaries, particularly those of senior professors, are high; retirement benefits are generous; SNFS research grants are easily obtainable. On the other hand, I do not share the view of ample opportunity for research. Minimum teaching loads for faculty at the University of Zurich, for example, are eight hours a week; an

excessive amount of time is spent at *ad hoc* committee, departmental, faculty and senate meetings, occasionally lasting past midnight; and a similar amount of time is spent in exchanges with hordes of administrators at all kinds of levels of the education ministry of the canton.

As noted by Steven Dickman on the same page, student/faculty ratios are extremely biased in some fields. Even more crucial, it seems to me, is that as a by-product of a lax or non-existent admission policy, the range in quality of students is also extreme. This, in combination with the large number of students, has led to a decline in the quality of research and teaching, approaching mediocrity at best in some departments.

The bending of rules by cantonal officials to allow foreign academics to buy housing without having lived in Switzerland for five years is certainly a nice gesture. By contrast, once arrived in the land of Alps, foreign academics are often treated as second-class fellows by their Swiss colleagues. I believe there may be an analogy here to the treatment of women as second-class citizens in Swiss society. In 1984, Frau Elisabeth Kopp was elected by her parliamentary colleagues into the executive branch of the federal government, a first in Swiss history. How about the faculty of one or the other Swiss university electing one of their foreign colleagues to a deanship or even a chancellorship? Recently, Kopp was forced to resign from her cabinet post. I hope that is where the analogy would break down.

WALTER LEUTENEGGER

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Obfuscation

SIR—The recent letter by C. A. Smith *et al.* (*Nature* **337**, 181–84; 1989) seems to be an example of the apoptotic misuse of the Greek language. As before with other Greek words, I was unable to establish an unambiguous meaning of apoptosis. *αποπτωω* is translated in the standard Greek–German dictionary as *verirren* (to go astray), so apoptosis might then be aberrant, and apoptosis could be translated by aberration. Using these meanings in the article, however, I was led astray: apoptosis as a process related to T lymphocytes, apoptotic as characteristic of these cells remained meaningless. The authors, however, seemed to like the word. But for a broad understanding of the article it is misleading. I am afraid that such use of cryptic Greek language by British scientists is a way of establishing barriers between them and others — deliberately. I wonder?

BERNHARD KLEINE

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Antarctic hero

SIR—I was appalled to see (*Nature* **336**, 417; 1988) that your Sydney, Australia, correspondent or your typesetter, or both, managed to spell the name of one of Australia's heroes as Sir Douglas Lawson rather than Sir Douglas Mawson. Any geologist who has ever handled a stromatolite knows his name (that includes me) and anyone with even the most meagre knowledge of Antarctic exploration is well aware of his exploits (that includes his great-grandniece, to whom I am married).

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More ways with neural networks

Ridding neural networks of some of their limitations seems to have suggested ways not just of accounting for the way that attentiveness can improve perception, but for human frailty as well.

PHYSICISTS, as eager as ever to believe that even biology is only applied physics, are naturally attracted to the notion that neural networks are a useful model for thinking processes capable of pattern recognition. One reason is that the formal description of networks is nicely mathematical. Another is that networks have already been used to solve other problems. An obvious, indeed an almost exact, analogy is that of the structures called spin-glasses, systems in which randomly distributed spins, magnetic or otherwise, interact with each other with a strength dependent on their random separations.

What could be more like the model of, say, the cortex, in which ON/OFF elements stand for neurons and in which the synaptic interconnections are described by coefficients whose magnitude determines the degree to which one neuron influences any other? Work on the spin-glasses was the first clearly to show that there is no unique equilibrium state of such a system but, rather, a large set of configurations, each of which represents a local minimum of the physical free energy.

In spin-glasses, these configurations are those in which the system may be trapped if the temperature is not great enough to allow random processes to discover and then to occupy another energy minimum with lower energy (which is the process of annealing). In neural networks, the same local minima correspond to the built-in patterns of the stimuli the network is capable of recognizing. Put crudely, a randomly chosen stimulus will first excite the network into a new state, which will then relax towards that built-in configuration to the stimulus most closely corresponds. In practice, this means that neural networks can recognize even very distorted versions of their built-in patterns.

But how well do the neural networks represent the real world of cognitive pattern recognition? While nobody expects utter precision from such inherently simple models, there are several respects in which they fail to correspond with everyday experience. All of us, for example, know that we can unconsciously, or even consciously, modulate the ease with which we recognize particular patterns by concentrating our attention on them: by what means do we adjust the coefficients representing synaptic strength in the manner this everyday observation implies? And what is to be said of the all-too-familiar situation that the cortex,

when presented with some arbitrary stimulus, can only tell us that it cannot make sense of what it has perceived — that no one built-in pattern seems more appropriate than any other?

Two researchers seem now to have been able to adapt the standard neural network model to these needs, suggesting how it may be possible for a working neural network to adjust its own noise level and thus the ease with which particular patterns may be perceived. As a by-product, they suggest how the same modification may allow neural networks quickly to recognize which stimuli are novel.

Maciej Lewenstein and Andrzej Nowak, respectively a theoretical physicist from the Polish Academy of Sciences institute and a psychologist from the University of Warsaw, take the acknowledged defects of existing neural networks as the starting-point for their new construction (*Phys. Rev. Lett.* **62**, 225; 1989). Much of their argument is stimulated by the Monte Carlo computational techniques applied, for example, to the calculation of the stationary states of spin-glasses: by flipping randomly chosen spins in an initial configuration, it is possible to move systematically towards a nearby local minimum of the free energy. But the argument is, mercifully, not just another exercise in computational simulation.

First, Lewenstein and Nowak seek to distinguish between two kinds of built-in patterns or memories, some of which are more 'strongly' memorized than others. The operational difference is that between deep and shallow wells in a free energy surface, but the authors say it is the kind of categorization that might be found in hierarchically organized memories. How to organize such an arrangement?

The underlying assumption in the analogy between neural network models and the real cortex is that both structures must 'learn' which patterns to 'memorize', which requires that neural networks should discover what pattern of coupling strengths between different pairs of elements will embody a learned pattern in a stable fashion — and that neurons in the cortex should similarly grow appropriate synaptic connections.

What the authors find is that their networks will acquire a number of poorly 'remembered' patterns if equal weights are assigned to them in constructing the coupling constants between pairs of elements and if the well-remembered pat-

terns are blessed with larger weights. Among other things, they find that the capacity of the network to recognize the poorly remembered states in an input stimulus is not impaired. But the unequal treatment does have the effect of reducing the number of the distinctive patterns the network may embody. In reality, there is no reason why the result (hierarchy) should not be advantageous in a real cortex.

The more striking innovation is that used for allowing the model neural network systematically to adjust its own precision, which is done by building into the equations rules that describe the way in which the noise level of the system is locally determined by the lability of the network during its dynamic evolution.

The noise in the system must be likened to thermal noise, and determines the likelihood that it will spontaneously migrate from one local minimum to another that is more stable. But under the influence of an external pattern, all the spins will be shaken up (neurons will be switched off and on), whereafter the system will evolve to the remembered pattern most nearly determined by the stimulus.

The rules allowing the analogues of attentiveness to be built into recognition by a neural network are recurrence relations relating the noise at one point in the pattern-seeking process to that at an earlier stage by means of quantities representing the numbers of elements in the network changing their state in the interval.

Those working in the field will no doubt be eager to get their hands on the details. For the rest of us, the qualitative implications may suffice. Thus a neural network in the process of homing onto a remembered pattern will, anthropomorphically speaking, do so with increasing confidence. But a network presented with a stimulus that cannot easily be recognized for what it is will change the states of a great many elements in hunting for a solution, the noise will as a result increase and the network may, in effect, be saying 'don't know'. There is also the possibility that the state of a network faced with a badly chosen stimulus will as a consequence be impelled into a noisy state and then driven by random processes towards the wrong attracting image state. In short, this adaptation of the standard neural network seems both to account for known psychological effects and for a good deal of human frailty — uncertainty and downright misjudgement.

John Maddox

Animal behaviour

The sight of deep wet heat

Michael F. Land

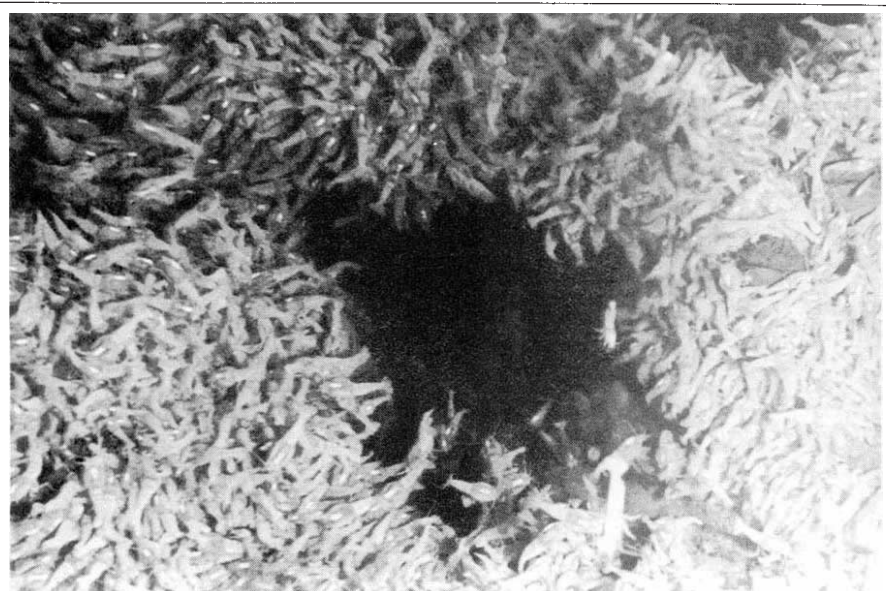
DAYLIGHT disappears at a depth of about 800 m in the ocean. There are simply too few photons left for eyes to capture them in numbers large enough to provide statistically usable signals^{1,2}. Below this depth most of the sparse fauna is eyeless. In the black depths of the Mid-Atlantic Ridge, 3.6 km deep, there are volcanic vents emitting very hot (350 °C) sulphurous water. Inhospitable as these sound, they are far from sterile. In particular they are inhabited by swarms of shrimp feeding on the sulphur-metabolizing microorganisms (see figure) that find the sulphide chimneys congenial. The strange thing about these shrimp is that they possess hugely developed structures that have much in common with eyes, as Van Dover *et al.* report on page 458 of this issue³, even

It is obviously a good thing for the shrimp to have a way of determining their proximity to a vent. Around the vent there is abundant food, but in it the shrimp would be instantly pressure-cooked. The question is whether or not the vents give off radiation that can be seen. I admit to a deep-seated reluctance to believe that columns of water can glow, related, I suspect, to my knowledge that water is for putting fires out. Nevertheless, the paper by Pelli and Chamberlain on page 460 of this issue⁴ has educated me. These authors treat the vent simply as a 350 °C black-body radiator, and work out how much energy there would be in the part of the spectrum to which the shrimp's visual pigment is sensitive. (Oddly, perhaps, the pigment is a normal blue-green sensitive

resolution is needed and optics are essential. But this may not be so for the detection of a single luminous source. The number of photons that can be captured and counted depends only on the area available to trap them, and this area can consist either of the pupil in an image-forming eye, or an area of naked retina as seems to be the case here. The naked-retina solution has the advantage of compactness; it is easy to fit a large detecting area into the carapace itself, whereas an eye of equivalent area would need to be large and three-dimensional. Provided only the crudest directionality was required, the eye's optics would in any case not be necessary.

If this shrimp, *Rimicaris exoculata*, were the only animal to have lost its optics while retaining the retina I would feel that the story presented in these two papers^{3,4} was complete. However, there are actually many other naked-retina eyes among deep-sea animals^{5,6}. These include other shrimp, isopods, amphipods, mysids and the famous fish *Ipynops murrayi* that turned up on the bottom of the sea at 3,500 m depth during the first deep-sea *Challenger* expedition of 1873. *Ipynops* has large, slightly concave retinas consisting mainly of rods, and covered simply by a cornea-like plate^{7,8}.

It is possible that all these animals live near hydrothermal vents, but this seems very unlikely. At these depths the only other use of the eyes would seem to be the detection of bioluminescence, but again, why abandon directionality when it would clearly be useful? The answer may be that these eyes (possibly including those of *Rimicaris*) are actually not quite as non-directional as they seem. A vertebrate rod, with a refractive index of 1.41, will trap light by total internal reflection over an angle of 36°, and a crustacean rhabdom of index 1.37 over 24°. In both cases, a curved array of these photoreceptors with no further optics could constitute an eye that would provide enough resolution for the rough direction of bioluminescent objects to be determined, as well as providing high sensitivity for their detection. It could be that we are dealing here not with naked retinas but with a new type of eye. □



R. exoculata swarming over the surface of sulphide chimneys at Mid-Atlantic Ridge hydrothermal vents. The bright patch on their backs corresponds to a novel visual organ described by Van Dover *et al.* on pages 458–460 of this issue. (Photograph by Andrew Campbell.)

though the usual spherical eyes of ordinary shrimp have disappeared. What might be the function of these strange organs?

There is a considerable amount of bioluminescent activity in the deep sea, including near sulphide chimneys, so it is possible that these peculiar eyes might detect that. But, as Van Dover *et al.* point out³, most animals that are known to use bioluminescence for communication have normal image-forming eyes. Indeed they must have, if they are to determine the directions of small sources of light. Rather than the detection of any conventional source of light, the authors suggest that these structures have a unique function: the detection of the sulphide vents themselves.

rhodopsin, not especially sensitive in the red.) After heroic calculations, involving the estimates of pigment density made by Van Dover *et al.*³, Pelli and Chamberlain conclude that the plumes would be detectable by an 'eye' of this kind, and at a distance of around 2.3 m for a 10-cm-wide vent. Pelli and Chamberlain also do the calculation for the human eye, and conclude that for us the luminance of the vents would be just below threshold — a result that squares with the initially damaging finding that dark-adapted observers in the Alvin submersible could not see them.

Why have the shrimp eyes abandoned their optics? Are such eyes actually better at certain detection tasks? For most vision,

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Nuclear physics

Engineering with quark matter

Charles Alcock

THE possibility that small drops of 'quark matter' might be stable was first put forward by Witten¹. More detailed calculations by Farhi and Jaffe² showed that the hypothesis was certainly plausible; also these authors named stable quark matter 'strange matter' because of the important role played by strange quarks, a name which has stuck. The strange-matter hypothesis has several interesting consequences, principally in astrophysics (for a review see ref. 3). Two new papers, that of Brügger *et al.* on page 434 of this issue⁴ and one by Madsen⁵, address the question of how much strange matter there is in our part of the Universe. And Shaw *et al.* on page 436 of this issue⁶ suggest a novel scheme for producing small lumps of strange matter, collecting and then growing the lumps; this is a proposal for engineering with quark matter.

Quark matter is a hypothetical phase in which quarks are not confined in individual hadrons such as the proton (which consists of two 'up' quarks and one 'down') and the neutron (one up and two down quarks), but are free to move about within the phase. Strange matter is a quark-matter phase which contains three flavours of quarks — up, down and strange — and a few electrons. By hypothesis, strange matter is absolutely stable, which means that its mass (energy) per quark is lower than that of iron (which is the most stable ordinary nuclear matter) and cannot decay without violating energy conservation (see figure). This hypothesis is consistent with phenomenological models of the strong interaction, but good first-principle computations of the mass per baryon are not available.

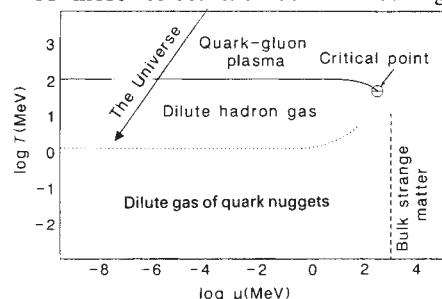
Because strange matter is a bulk phase, it might be found in lumps of atomic mass A in the range 10^2 – 10^{57} , but with atomic number (charge) Z much less than $A/2$. These positively charged lumps are often known as quark nuggets. They behave chemically as ordinary atoms with the same Z and are, roughly speaking, monstrous isotopes. A quark nugget may grow by absorbing neutrons; charged particles such as protons are not readily absorbed because of Coulomb repulsion.

For theorists, not being able to recognize the true ground state of the strong interactions (is it iron or strange matter?) is very disturbing. It makes sense, therefore, to search for quark nuggets or use experiments to settle the question. Madsen⁵ proposes that observations of pulsars should help. Conventionally it is suggested that neutrons are the stable form of matter in the enormous gravitational field of

these collapsed stars, but some argue that strange matter is. For his proposal, Madsen adduces the conclusion of Alpar⁷ that the radio pulsars which exhibit glitches (abrupt, very small period reductions) followed by a few months of relaxation) must be made of neutron matter, not strange matter. This conclusion rests on the success of neutron-star models for the glitch phenomenon and the absence of a model for glitches in the context of strange matter. Because strange matter absorbs neutrons, one seed of strange matter will convert an active neutron star into a 'strange' star. Madsen's analysis rests on the rate at which these seeds would be accreted from the interstellar medium.

Should they exist in the interstellar medium, quark nuggets will rain steadily onto stars, where friction slows them down. Thus a nugget can become bound to a star. If the star is a neutron star, the nugget will act as a seed which will grow until the entire star is converted to strange matter. Furthermore, if the star is the progenitor of a neutron star (a high-mass normal star which eventually explodes as a supernova, compressing its core to a neutron star), then any nuggets it captures will be present later when the neutron star is made. Madsen shows that this latter process is the most interesting and places limits on the number of quark nuggets in the interstellar medium. This is a model-dependent limit, of course, but it does show how to approach issues of fundamental physics using astrophysics.

A more direct method for seeking



As shown in this hypothetical phase diagram, strange matter is a high-density, low-temperature phase (density increases with chemical potential μ). At high temperature, strange matter evaporates into ordinary hadrons (dotted line), much as a solid sublimates into vapour on heating; this transition is not a phase transition. At higher temperature, the hadron gas undergoes a phase transition (solid line) to a hot gas of quarks and 'gluons'. Cosmic or experimental production of strange matter occurs by producing the hot quark-gluon plasma with the hope that rapid cooling will trap portions of the matter in the cold, condensed phase. A detailed mechanism for accomplishing this has not been proposed. (Temperature T in megaelectron volts; $1 \text{ MeV} \approx 10^{10} \text{ K}$.) (From ref. 3.)

quark nuggets has been used by Brügger *et al.*⁴, who have adapted Rutherford scattering, bombarding material samples with beams of ^{238}U and ^{208}Pb nuclei. Any back-scattering of the beams by angles greater than 90° indicates the presence of scattering centres (the nuggets) more massive than the projectile nuclei.

Brügger *et al.* applied this elegant technique to various natural samples (including a meteorite) and derive upper limits to the abundance of quark nuggets: 1 per 10^{10} for nuggets with $A \approx 10^3$ down to 1 per 10^{14} at $A \approx 10^7$. The technique is less readily applied for higher atomic number because the electrostatic potential of the nuggets is not a simple Coulomb form.

Shaw *et al.*⁶ propose a much more direct approach to the study of strange matter. They propose that high-energy heavy-ion collisions of the kind studied in present CERN and Brookhaven National Laboratory experiments might make small quark nuggets. They further describe a scheme for isolating, slowing down and storing nuggets which are produced in the experiment. Any nugget which is captured may then be "grown" by the addition of low energy neutrons. This growth phase releases of order 20 megaelectron volts of energy per captured neutron — potentially a useful source of power, as this is nearly 10 times the energy yield per neutron in a conventional fission reactor. In this regard Shaw *et al.* are proposing a first attempt at engineering with quark matter.

There are uncertainties in this proposal. First, it is not clear that the CERN and Brookhaven experiments do indeed make a quark phase. Second, this phase will contain plenty of up and down quarks but strange quarks are produced only as $s\bar{s}$ pairs, and the strange quarks must separate from their anti-strange counterparts. Third, strange matter is a low-temperature phase and these experiments produce a very hot plasma (see figure). And fourth, the isolation scheme proposed looks for negatively charged nuggets, whereas strange matter is almost certainly positively charged in its most stable form.

Despite these reservations, the proposal does not seem to be an expensive addition to experiments that are already in progress. As the payoff in physics would be so large if strange matter were detected, the ideas should be further explored. And the new engineering ideas raise interesting long-term prospects for energy generation. □

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Peptide structure

Beyond transcriptional events

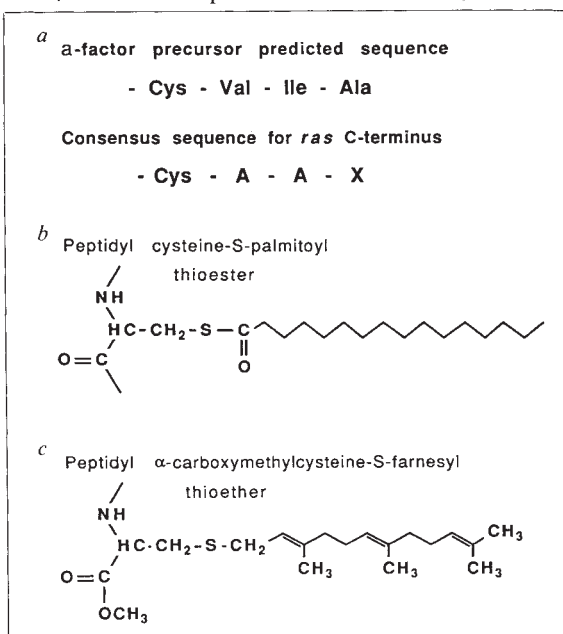
Anthony M. Treston and James L. Mulshine

PROTEIN chemistry, the demise of which was predicted in a News and Views article¹ 10 years ago, is still an essential technique for determining protein structure. Robert Anderegg and colleagues in the December 1988 issue² of the *Journal of Biological Chemistry*, for example, report the structure of a yeast mating pheromone using conventional protein-chemistry techniques. Their results demonstrate that, given the present lack of understanding of the mechanisms and control of post-translational processing, the prediction of processing events solely on the basis of nucleic-acid sequence data is speculative in the absence of supporting protein chemistry data.

The traditional approach for determining the structure of peptides and proteins has been through what could be called classical analytical methodology, including purification, derivation, hydrolysis and amino-acid characterization. Recently, however, these approaches have lost popularity because of the speed and precision of sequencing nucleic acids, enabling the prediction of the order of amino acids directly from DNA and RNA sequences. This 'molecular biology' approach to protein chemistry, although accurate, is confined to deriving the linear order of common amino acids in polypeptides.

Many peptides and proteins, however, contain amino acids which have been modified in various ways after translation from their messenger RNA, and these modifications cannot be derived directly from the primary structure of the gene. These post-translational modifications are essential for the biological function of the mature polypeptide, and both the types of modifications and the mechanisms by which they occur are apparently highly conserved across species ranging from yeast to man. The conservative nature of these modifications has allowed the determination of precursor protein consensus sequences which become the substrates for the post-translational processing reactions producing the mature molecule. The ability to predict polypeptide sequence from the primary nucleic-acid structure, coupled with knowledge of characteristic processing mechanisms, can in theory lead to the prediction of the mature processed form of polypeptides directly from a nucleic-acid sequence. But Anderegg *et al.*² demonstrate that a consensus sequence alone is not necessarily a reliable predictor of the structure of a mature peptide.

The nucleic-acid sequence encoding the mating hormone called a-factor from the yeast *Saccharomyces cerevisiae* has been known since 1985. The predicted precursor prohormone has at its carboxy terminus the sequence . . . Cys-Val-Ile-Ala, consistent with the consensus sequence for the acylation of the carboxy terminus of *ras* genes (a in the figure; sequences summarized in ref. 3). The identity of these sequences suggests that yeast a-factor should undergo the same post-translational processing as the yeast *ras*-related proteins. Furthermore, to



exert their activities as regulators of cell proliferation, *ras* proteins must be inserted into the cellular membrane, and a similar mechanism has been postulated to be necessary for a-factor activity³. Membrane insertion occurs only for *ras* proteins that have been modified at the cysteine residue near the carboxy terminus by conversion into a thioester of palmitic acid⁴. These considerations all point towards identical post-translational modifications for *ras* proteins and a-factor, that is, conversion to a palmitate cysteine-thioester (b in the figure).

In fact, a yeast gene has been identified on the basis of being involved with the processing of both *ras* and a-factor. *RAM* (for *ras* and a-factor maturation function) was described⁵ in 1986, and is essential for sexual activity of a-type haploid yeast and for the palmitate esterification and membrane localization of *ras* proteins. It was originally proposed that the *RAM* gene encoded an acyltransferase, with the *de facto* corollary that both a-factor and *ras*

have the same cysteine-thioester modifications. A gene called *DRP1* has now been shown⁵ to be responsible for a post-translational processing step occurring before fatty-acid alkylation of *ras*⁵. *DRP1* turns out to be identical to *RAM*.

The data of Anderegg *et al.*² now unequivocally show that the cysteine modifications of *ras* and a-factor are not identical, or even similar. These authors purified a-factor from *S. cerevisiae* and established by proton nuclear magnetic resonance and mass spectrometry that the biological activity of a-factor results from a pair of closely related post-translationally modified peptides with a carboxy-terminal α-carboxymethylcysteine-S-farnesyl ether (c in the figure). Farnesol, a branched-chain, polyunsaturated hydrocarbon alcohol, is best known as an intermediate in the biosynthesis of cholesterol and related molecules. The presence of a farnesyl moiety as a cysteine-S-thioether *per se* is not unusual, as it is known to be a component of peptide pheromones from other fungi. Not only is the alkyl group of a-factor an unsaturated branched-chain alcohol, whereas the *ras* protein has a saturated linear fatty acid moiety, but the chemical linkage is different in each case; a thioether for a-factor and a thioester for *ras*. These findings are totally incompatible with the predicted identical modifications postulated on the basis of identical carboxy-terminal precursor sequences.

In retrospect, the incorrect prediction of the nature of the a-factor modification and the identity of the protein encoded by *RAM* were caused by reliance on the 'molecular biology' approach to predicting peptide structure. The *RAM/DRP1* gene described by Powers *et al.*³ is obviously not an acyltransferase acting on both *ras* and a-factor. It seems more likely that *RAM* encodes a carboxypeptidase or endopeptidase responsible for removal of the tailing A-A-X amino acids of both precursor molecules to provide a free cysteine for modification. Alternatively, *RAM* could encode an enzyme responsible for the methylesterification of the α-carboxy terminus of both peptides. The assignment of a function for *RAM* is an illustration of the need for direct determination of peptide structures to resolve the nature of proposed post-translational modifications. □

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Biochemistry

A protein with many functions?

R. B. Freedman

A QUIZ for the New Year. What is the connection between: (1) a gene product associated with visual transduction in the fruitfly *Drosophila*; (2) the major high-affinity binding protein in vertebrates for the immunosuppressive drug cyclosporin; and (3) an enzyme catalysing a rate-determining isomerization in protein folding *in vitro*? The answer, in two papers on pages 476 and 473 of this issue^{1,2} and in a forthcoming paper³, is that they seem to be the same protein. What this identity means will take some time to work out, because the protein, from whichever direction it is defined, is not yet securely identified with any specific physiological function. But the fact that it catalyses the *cis-trans* isomerization of peptide bonds involving the prolyl residue — the rate-determining step for the refolding of some proteins from the unfolded state — makes it tempting to look for crucial protein-folding processes in the immune response and in visual transduction.

Fischer *et al.*¹ and Takahashi *et al.*² in their papers in this issue have reached this point by asking essentially the same question. Both groups were attempting to characterize further the peptidyl-prolyl *cis-trans* isomerase (PPI) which the former group has identified and shown to be an abundant cytosolic protein⁴. PPI catalyses isomerization of the stereochemistry about the imide bond in the sequence -X-Pro-, both in small model peptides and in proteins, and in some proteins the result is catalysis of folding from the denatured to the native state.

Fischer *et al.* have now identified¹ the 38 amino-terminal residues of porcine PPI using automated amino-acid sequencing. They find that this sequence is identical to the published amino-terminal sequences of cyclophilin from human and bovine sources. Cyclophilin was identified in 1984 as an abundant and widespread mammalian protein which binds with high affinity to the fungal product cyclosporin A, a cyclic peptide of 11 residues which is a powerful suppressor of the immune response. Because of the similarity of amino-terminal sequences, and of other properties such as molecular mass and amino-acid composition, Fischer *et al.* propose that PPI and cyclophilin are identical.

This proposal is confirmed by Takahashi *et al.*², who performed a complete protein-sequence analysis of PPI based on automated sequencing of peptides produced by trypsin, cyanogen bromide and V8 protease. Their results show that porcine PPI is identical in sequence to bovine cyclophilin and almost identical to human and

rat cyclophilin, whose structures have been derived from the complementary DNA sequence.

Both groups^{1,2} have pursued this surprising result to examine functional relationships between the enzymic activity of PPI and the binding activity of cyclophilin. They point out that previous results show both activities to be heat-sensitive, abolished by trypsin treatment and sensitive to modification of -SH groups. They now find that the presence of cyclosporin A inhibits the isomerase activity of PPI/cyclophilin and that the isomerase activity towards both peptide and protein substrates is completely inhibited by cyclosporin A at concentrations below 1 μ M. Fischer *et al.* estimate an inhibition constant of below 10 nM, which is in the range reported for the dissociation constant of cyclophilin for cyclosporin A, as measured in studies of the direct binding. It is thus possible that PPI/cyclophilin is not just a high-affinity binding site for cyclosporin A, but is also the site of the drug's primary biological effect — the inhibition of the enzymic activity of PPI.

Where does visual transduction in *Drosophila* come in to all this? Charles Zuker and colleagues have been analysing the mechanism of visual transduction by characterizing mutations which affect this

process in *Drosophila*. They constructed a library enriched for genes encoding proteins preferentially expressed in the adult visual system, and hybridized clones isolated from this library to polytene chromosomes to determine their positions on the cytogenetic map. One of their clones (λ 322) maps to the position 21D4-E2, within the position of a known visual transduction gene *ninaA* (located at position 21D3-E2 by conventional genetic mapping). The phenotype of the *ninaA* mutant is a drastically reduced level of the visual pigment rhodopsin, but there is no reduction in opsin expression. The mutation is therefore thought to affect post-translational modification of opsin or its degradation. By any criterion this is an interesting mutation, and it would be intriguing to know the nature of the *ninaA* gene product. Zuker and colleagues³ now present the sequence of their clone λ 322, and the inferred sequence of the *ninaA* gene product. This protein comprises 237 residues, and its central 180 residues are almost identical to cyclophilin/PPI; bovine cyclophilin/PPI has more than 40 per cent of the amino-acid residues, in common with the *Drosophila ninaA* protein, and conservative substitutions account for more than 30 per cent of the remaining residues. Given the taxonomic distance involved, the similarity is striking.

Taken together, the new reports identify an intriguing protein or protein family. In vertebrates, cyclophilin is clearly the main high-affinity binding site for cyclosporin A, and is thought to be its primary site of action; this action is not well-defined, but



FLUORESCENT tracing of motor axons in the developing hindbrain of the chick, contributing to evidence that growing nerves in this region respect segmental boundaries that may be analogous to those that define the body pattern of *Drosophila* during development (see the paper by S. Lumsden and R. Keynes on page 424 of this issue). The boundaries of the segments are preferentially stained with anti-laminin antibodies while the more central regions show preferential expression of the embryonic neural cell-adhesion molecule N-CAM. Strikingly, similar boundaries seem to be observed in the early embryonic mouse hindbrain by the expression of the zinc-finger gene *Krox-20*, which is expressed in alternating segments in the same region (see the paper by D. G. Wilkinson *et al.* on page 461 of this issue). □

it appears that cyclosporin A interrupts an early event in the antigenic activation of T-helper cells, leading to production of lymphokines. The new work shows that this protein is identical to the enzyme prolyl-peptidyl *cis-trans* isomerase, but the physiological function of this enzyme is not known. The presence of a closely related protein modifying the properties or stability of the visual pigment in insects raises a whole further set of questions. The power of gene and protein sequencing and the use of sequence databases have here connected three apparently disparate

lines of work¹⁻³. Now, more conventional studies on the properties of PPI, and on the roles of cyclophilin in the immune response and of the *ninaA* product in the visual system, will be needed to make sense of that connection in functional terms. □

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Optical computing

Polymers that make light work

Paul Calvert

THE prospect of computing with light rather than electricity is attractive because light pulses travel faster in glasses than electrical pulses in metals, and light transmissions are less vulnerable to interference from outside, or from cross-talk between adjacent wires. At a recent symposium*, workers showed that they are close to developing polymers suitable for use in applications such as optical switches.

To make an optical computer, one needs ways of switching light beams on and off, or of diverting them between different paths. This requires a material whose refractive index changes either as a function of light intensity or as a result of an applied electrical field (see figure). The first effect would make it possible to build an all-optical computer, the second to use light to couple different parts of an electronic computer — an internal expressway. Conjugated organic molecules (with alternating double bonds) have large values of the important nonlinear optical coefficients and, although the simple compounds tend to give poor films, the equivalent polymers are readily processed into thin films for integrated optics.

The basic equation governing the nonlinearity is

$$P = \chi^{(1)}E + \chi^{(2)}E^2 + \chi^{(3)}E^3 \dots$$

where the polarization of the material P depends on the applied electrical field E to a series of powers. E can also be the oscillating electric field of light, in which case the first coefficient $\chi^{(1)}$ is related to the usual refractive index. The subsequent coefficients tend to be very small and so become

important only with intense light, such as emitted by lasers. Although I will discuss them as simple coefficients, $\chi^{(1)}-\chi^{(3)}$ are tensors whose values are dependent on the directions of the input and output fields; $\chi^{(2)}$ is a third rank tensor (a three-dimensional array) and workers in the

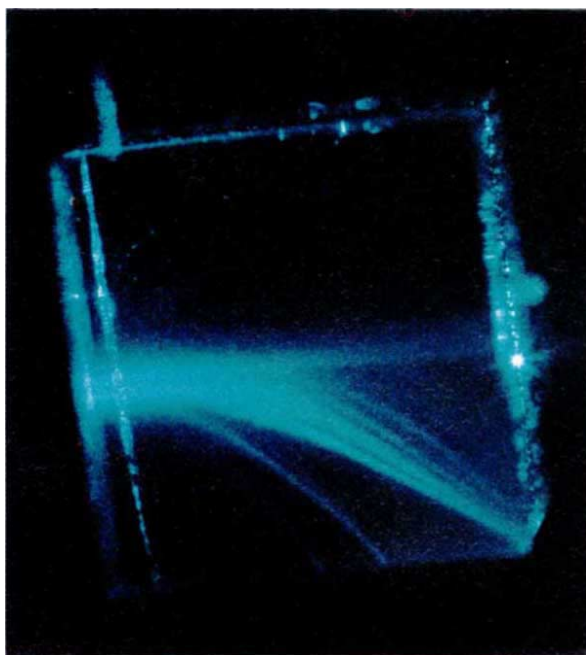
travels more slowly through this than through air alone, as each pipe stores some vibrational energy and then gives it back. As the sound level is increased, the pipes become nonlinear and produce more overtones. The amount of second and third harmonic depend, through $\chi^{(2)}$ and $\chi^{(3)}$, on the properties of the pipes and their arrangement. Effects of $\chi^{(2)}$ will cancel out in a random or centrosymmetric arrangement of pipes.

The materials' requirements are: a big coefficient $\chi^{(2)}$ or $\chi^{(3)}$, no absorption of the light and little scattering so that as little light as possible is lost in each device, a fast response and easy processing into thin films. The material to beat is lithium niobate, which may turn out to be as ubiquitous in integrated optics as silicon is in electronics. At present it is damaged by the high intensities needed for all-optical switching, and although electro-optic switches can be made (see box), they are rather large because a long path between the electrodes is needed to produce sufficient effect on the light, and the response is rather slow. For a fast response, the effect must depend purely on the molecular electronic structure, anything involving atomic or molecular motion being too slow.

Crystalline, asymmetric, organic materials exist with very high $\chi^{(2)}$ coefficients, for example 2-methyl-4-nitroaniline. The problem is that the crystalline films tend to scatter light at the grain boundaries, they are weak and they evaporate. Such compounds can be incorporated into amorphous polymers to give a relatively tough transparent film, but $\chi^{(2)}$ is zero unless an asymmetric structure can be induced. This can be done by applying a strong field across the film to align the active groups, but the effect is still weak. Working electro-optic devices have been made from these polymers. At the recent meeting, J. Stamatoff, of Hoechst-Celanese (which leads the field) described a polymer electro-optic switch that worked at 7 volts but was 1.3 centimetres long. That is enormous by current computer standards.

Another approach is to forget $\chi^{(2)}$ and look for a large $\chi^{(3)}$, for which no molecular alignment is needed. Favoured materials are

highly conjugated polymers. The extensive π -orbital systems make these the electronic equivalent of a rubber organ pipe and large $\chi^{(3)}$ values (around 10^{-10} e.s.u.) have been measured for both polyacetylene¹ and the polydiacetylenes². The chief problem with polyacetylene is that it is black and so absorbs most of the



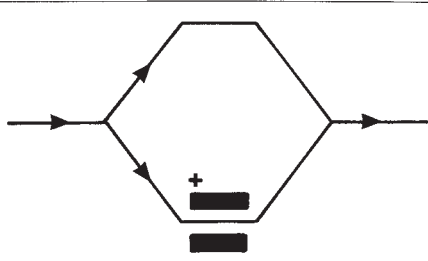
Bending light. The electric field of a laser beam induces changes in the refractive index of a barium titanate crystal which in turn bend the laser beam. Increasing the field intensity changes not the degree of bending, but rather the onset time for the effect, which is non-zero. (Courtesy of Malcolm Gower, Rutherford Appleton Laboratories.)

field are not yet ready to tackle the problem of defining the whole set (up to 27) of coefficients.

Consider the molecule as an organ pipe. It vibrates when excited by a beam of sound, even if the frequency is not the natural one of the pipe. A crystal is a room full of such pipes, regularly spaced. Sound

* Materials Research Society, Boston, 28 November-3 December 1988.

Pictured right is a Mach-Zehnder interferometer, one of several proposed switching devices⁷. Light is guided through a 1-micrometre channel between walls of slightly lower refractive index. The beam is split between two legs one of which has two electrodes above and below. Applying a voltage changes the effective refractive index in that channel so that the light slows until it is exactly out of phase with the other leg: when the beams are recombined, nothing emerges. This can convert electronic data to light pulses for transmission between parts of a computer that are too far apart for electronic conduction to occur rapidly. A similar logic device could have one leg intrinsically nonlinear such that the light in that leg slows as the intensity increases until it becomes out of phase with the other leg. This could then be part of an all-optical computer. The small channel size allows very high light intensities to be reached with relatively low total powers.



signal, but it is also intractable and unstable. As $\chi^{(3)}$ increases with the extent of conjugation to the fifth power, long conjugated chains should have huge values. Unfortunately these systems also absorb light.

The polydiacetylenes are coloured but transparent at long wavelengths and some can be cast as films from solution. They can also be prepared as Langmuir-Blodgett films (similar to soap films), but these tend to include light-scattering defects and the optically active groups are diluted by the soluble hydrocarbon tails needed at the processing stage.

Polybenzobisthiazole (PBT) also has a $\chi^{(3)}$ of around 10^{-10} e.s.u. (ref.3; Prasad, State University of New York, Buffalo). This is one of a group of rigid rod polymers developed from work at Wright Patterson Air Force Base on high-temperature polymers. Polyphenylenevinylene is another black, conjugated, conducting polymer and has $\chi^{(3)} = 3 \times 10^{-10}$ e.s.u. when drawn into an oriented film.

The polysilanes, based on a silicon backbone rather than carbon, are an interesting possibility^{4,5}. For polymethylphenylsilane $\chi^{(3)} = 10^{-12}$ e.s.u. when measured with 1.06-micrometre light and decreases at longer wavelengths as the third harmonic is enhanced by resonance. Polysilanes are clear and can be easily made as films. They would be expected to have just a σ -bond system similar to polyethylene and no significant nonlinearity. The effect seems to arise from delocalization of electrons along the silicon-chain backbone which allows enough 'softness' to respond to the optical input. There is some evidence for this delocalization in the optical spectrum⁶. Also new data show that the response is very fast (taking less than 3 picoseconds) and is therefore due to electronic motion rather than molecular motion or thermal effects⁵.

One expects the nonlinearity of an organ pipe to increase as the resonant frequency is approached and it starts to shake vigorously. In molecules resonance is usually associated with absorption and so is to be avoided. If the aim is to generate third-harmonic light, there should be no

absorption at either the fundamental frequency or at the third harmonic. But to obtain a simple change of refractive index absorption at the third harmonic can enhance $\chi^{(3)}$ without detracting from the transmission of the fundamental. In general the measured $\chi^{(3)}$ rises as the fundamental wavelength is reduced until the third harmonic approaches an absorption band. In other words, absorption seems to be the price of a high $\chi^{(3)}$. Much attention was given at the meeting to molecular orbital calculations of $\chi^{(3)}$, which now can get within an order of magnitude of the right answer. We may be approaching a point where some useful prediction can be made of good structures.

Large inorganic crystals are already used for frequency doubling and tripling for laser spectroscopy, but these materials are difficult to adapt to small waveguides. Organic materials with extended conjugation offer large nonlinear coefficients and rapid switching when compared to inorganic materials. Polymers can be stable, easily formable and can (with difficulty) be made to high optical transparency. Whether these properties can all be combined in a single system remains to be seen. One view is that the stringent requirements of purity and stability in a computing system will prove to be too much for organics, as seems to be the case with conducting polymers. Another view is that opto-electronic transducers have been functioning effectively in chloroplasts and retinas for a long time and there should be no problem. □

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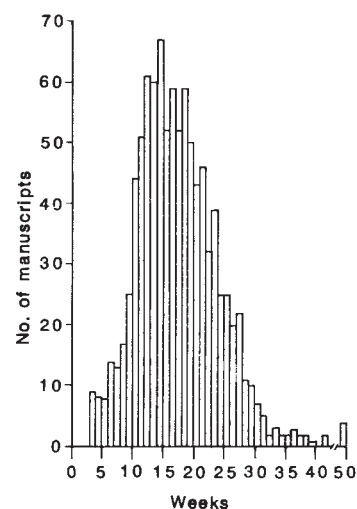
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Nature statistics

IN 1988, about 6,000 unsolicited manuscripts were submitted to *Nature* for publication as Letters or Articles — almost exactly the same number as in 1987. About 35 per cent were sent to our Washington office rather than to London — there is no preference. About 30 per cent dealt with physical sciences rather than biological sciences. As usual, submissions peaked in the summer months to coincide with the period when potential referees (and *Nature* staff) are most likely to be away.

Of the submitted papers, just over 1,000 were published, 400 in the physical sciences and 600 in the biological sciences. If 1988 is no exception to the pattern of the past few years documented by the *Science Citation Index*, 30 of the 600 biological papers will be among the 100 life-science papers published in 1988 most cited by the end of 1989.

On the average, the delay between receipt and the appearance in print of the papers accepted for publication was just over 4 months. (Papers not accepted for publication are returned to their authors much more quickly.) As the figure shows, 30 or so papers, mostly on high-temperature



superconductivity, were published within 6 weeks of submission, but an equal number took more than 8 months to appear in print — sometimes because they were overgenerously granted the original date of submission despite long delays during substantial revision. The median time between receipt and acceptance was 10 weeks, and from acceptance to publication was 7 weeks.

Naturally, we hope that publication will be even faster in 1989. One helpful development is the increasing use being made of telefax machines. For example, the proportion of referees' reports sent to our Washington office by that means increased from 8 per cent in June 1988 to 35 per cent in December. During 1989, we expect a further increase in the use of fax machines and some growth in the use of electronic mail to deliver reviews of manuscripts. □

Neuroscience

Cognitive control of movement

Roger Lemon

RECORDING from single cells in conscious animals has the potential to provide direct insights to the working brain. Investigation of cerebral events associated with movement using this approach has shown that cells in different areas of the cerebral cortex are involved with different aspects of movement performance¹. Now, Georgopoulos *et al.*, in the 13 January issue of *Science*², make the exciting claim that studying the activity in populations of cortical neurons can allow visualization of the cognitive processes preceding movement. These new results raise important issues about the central processing that occurs within the motor cortex before movements are executed.

Georgopoulos *et al.* use mental rotation³ as an experimental test of cognition in a rhesus monkey. In this procedure, the monkey is rewarded for making a movement at an angle to a target light whose position is changed from trial to trial. This

process has been extensively investigated in humans^{4,5}, and it has been suggested that subjects solve the problem by mentally rotating an imagined movement vector from the direction of the stimulus to that of the required movement.

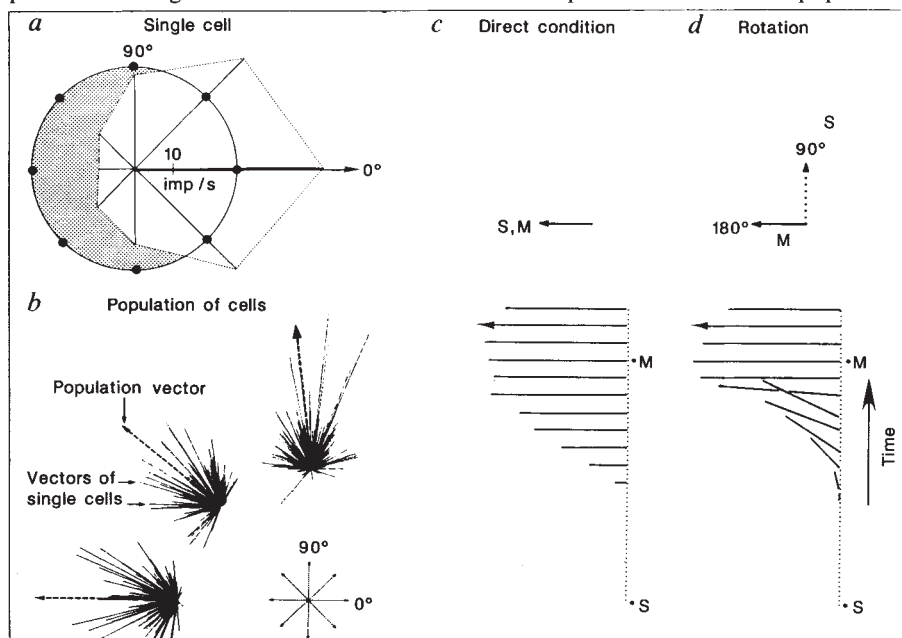
Georgopoulos and his collaborators have already shown⁶ that the direction of movement is represented in the monkey motor cortex by the neuronal population vector (see figure legend). This concept is attractive because it concerns the contribution of a whole population of cells, rather than of individual neurons. In their new study², Georgopoulos *et al.* recorded the activity of single cells in the motor cortex during performance of the task and computed the behaviour of the population during the reaction time. They find that the information content of the population vector is greater than would be required to explain reaching performance in humans⁷.

An important feature of the population

vector is its predictive value. Most neurons in the motor cortex are active before the movement is performed, and the direction of the population vector is a good indicator of the direction of the upcoming movement. Georgopoulos *et al.*² trained the monkey to move either directly towards any one of eight target lights (*c* in the figure) or at 90° in an anti-clockwise direction to the light (*d* in the figure). When the monkey moves directly to the light, the population vector points towards the direction of the movement from about 125 ms after the target was illuminated; in the rotation condition, the vector first points in the direction of the stimulus light, and then begins an anti-clockwise sweep until, at about 225 ms, the vector points in the direction of the required movement.

Georgopoulos *et al.* claim that this rotation of the vector is the neuronal equivalent of the cognitive operation carried out by human subjects performing the same task. They point out that anticlockwise rotation of the vector (through 90°) is much quicker than in the opposite, clockwise direction (270°) and allows the monkey to solve the task quickly. The speed of vector rotation in this overtrained monkey was 732° per second, which is somewhat faster than the speed at which human subjects can perform the task³. Remarkably, the reaction time was the same for both conditions (260 ms after the target light was switched on). Thus in the rotation condition, only 35 ms elapsed between attainment of the 'correct' direction by the population vector and the onset of the appropriate arm movement.

Hierarchical notions of movement control imply that other, 'higher' brain structures, which project to the motor cortex, are involved in the cognitive operations needed to solve the mental rotation task. But the new results show that motor-cortex activity reflects these operations apparently throughout the preparatory period, and that the motor cortex does not receive only the final command to move (for if it did, the vector would not rotate, but it would already be pointing in the appropriate direction when it appeared). It is perhaps surprising to find evidence for these operations in the motor cortex, which has long been thought of as an executive structure in



Neuronal activity in the monkey motor cortex during directional reaching. *a*, The monkey is trained to move a lightweight handle from the centre of a table to one of eight target lights (dots) located at the edge of the working surface at 45° intervals. **Single cells** discharge for reaches in all eight directions, but are most active for one preferred direction (length of lines in *a* indicate mean firing rate during the reach). *b*, The broad 'directional tuning curves' of single cells make it unlikely that individual neurons code precise movement direction. But **populations of cells** could: *b* shows the vector contribution of the activity of a large number of single neurons; line length indicates discharge frequency. The **population vector** (dotted line) can be thought of as the sum total of discharges or 'votes' contributed by all the directionally tuned cells in the population; most votes will be cast by those neurons for which the required movement is in their preferred direction. The population vector points in the direction of the movement⁶. *c*, *d*, Temporal progression of the vector from the time the target light is switched on (*S*) to the onset of the reaching movement (*M*). The cells first become active about 125 ms after *S*, and movement begins at 260 ms. For the condition in which both stimulus and movement are in the same direction (*c*), the vector points in the required direction at all times. For the alternative condition, where the monkey must move in a direction of 90° to the stimulus (*d*), the vector first points in the direction of *S*, then rotates until, at about 225 ms, it is pointing at *M* (from refs 2, 6).

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movement control, an idea enshrined in the use of the term 'upper motoneuron' in clinical neurology.

The functional linkage of the cells sampled by Georgopoulos *et al.* to the muscles producing the reaching movement is unknown. The weighting given to all cells in the population vector is equal⁶, although it is known that some of these cells are probably interneurons and others are output neurons, projecting to the brainstem and spinal cord. It is also paradoxical that the time delay between discharge of even these output neurons

and the onset of muscle activity can be more than 100 ms, yet the descending pathways from the motor cortex to shoulder muscles can be traversed in a tenth of that time⁸. Discharge of these cells can also be dissociated from movement, because the introduction of a waiting period into the experimental protocol allows the vector to be seen without movement⁹. □

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Cosmology

The elusive anthropic principle

Marek Abramowicz and George Ellis

THE anthropic principle is a modern attempt to deal with a question as old as mankind: why are we here? It is posed in the particular sense: why does the Universe seem to have special and very peculiar properties supporting our existence? Is this by pure chance, or is there an underlying reason, a purpose, perhaps a special design? Although the participants at a recent meeting* were hard pressed even to agree on what is the appropriate way to approach the question, there was one proposal that observations of the homogeneity of the Universe could be used as a concrete test of a particular variety of the anthropic principle.

The notion of an anthropic principle arises from the peculiar circumstances that must occur for life to evolve during the lifetime of a universe. For example, the natural constants in our Universe not only allow for the existence of stable atoms from which matter is formed, but also (together with proper initial conditions of the Big Bang) lead to the formation of galaxies and stars, apparently vital features of life. The task that those interested in the anthropic principle have set themselves is to reformulate, in a useful way, the questions of whether this a fortunate coincidence, inevitable or even just to be expected.

It was apparent from the discussions that even this raises considerable controversy. Some participants maintained that the whole issue is not a real scientific question; others that it is a key issue in cosmology. It was clear, particularly during informal discussions, that this controversy has a philosophical as well as a scientific nature: opinions are based not only on the speakers' scientific knowledge or extrapolations thereof, but also to a great extent on their *weltanschauung* (world view).

L. Gratton (Rome University) was one

of the few who argued that the anthropic principle is meaningless. This was an attack on the strong version of the principle rather than the weak one (see below). F. Hoyle (University of Wales) argued that far from being meaningless, the principle supports the steady-state theory of the Universe; and G. Coyne (Vatican Observatory) argued that it supports (or at least is congruent with) the existence of God. Although these, and several other, philosophical interpretations were all analysed extensively, the main debate at the meeting took place on more physical grounds, between the supporters of the strong anthropic principle and those of its weak version.

The strong anthropic principle claims that the Universe must admit intelligent life, not by chance but of necessity. The weak principle stresses that we, as intelligent observers, may see (and do see) some apparently very special properties just because of the selection effect: intelligent life can only develop in particular places and at particular times. The issue, on this view, is to determine what restrictions this implies. Most participants came out against the strong principle and for the weak one. Interestingly, J.D. Barrow (University of Sussex), co-author with F.J. Tipler of the stimulating book *The Anthropic Cosmological Principle* (Oxford University Press, 1986), seems to have abandoned the strongest version expressed in that book, the final anthropic principle: not only must life come into being in the Universe, but it must then persist to the very end.

B. Carter (Paris Observatory), largely responsible for the renewed interest in the theme, was mainly concerned to argue the indisputable nature and power of the weak version of the principle. He points out that some appearance of a purposeful direction in biological evolution is entirely predictable (without invoking any mysterious design mechanism) provided

one bears in mind that in addition to the darwinian effects of natural selection one should also take into account the anthropic effect of self-selection; our observations are biased by the fact we live in a Universe where intelligent life has indeed come into being. Our failure to observe the type of alternatives in which intelligent observers are proscribed, self-evidently cannot mean that there is no chance of such alternatives occurring. Their existence is tautologically unobservable.

H. Reeves (Saclay) developed implications of this theme, looking at conditions for the emergence of complex structures in the Universe (a necessary pre-requisite for the emergence of life). The emphasis in this approach is very much a physical one, relating to the formation of such complex structures according to the known laws of physics. Thus both Carter and Reeves played down the question of what would happen in other universes with other laws of physics (the kind of issue raised in discussing the strong version).

Perhaps the strongest defender of a version of the strong principle was D.W. Sciama (International School for Advanced Studies, Trieste) who argued that there are three possibilities: life can occur through pure chance; because of some purposive design (God); or because all possibilities occur in some ensemble of universes that includes those where life can occur and others where it cannot. Sciama's thesis is that what is not forbidden is compulsory; that is, all logically possible universes must occur, disjointly from each other. In a sense this incorporates into cosmology Dostoevsky's remark: "If there is no God, everything is allowed."

The most surprising part of Sciama's argument is a suggestion that, although the other universes are all causally disconnected from the one we live in (and between themselves), there is a possible observational test to support the hypothesis of their real existence. Essentially, his argument is that if all logical possibilities occur, then our Universe should be no more special than needed; another universe with only slightly different properties would also be sufficiently special to support intelligent life.

Thus some of the properties of our Universe should be random in the above sense, or at least not particularly special. Hence we expect to find, with accurate enough measurements, some asymmetries in the Universe that do not accord, for example, with Penrose's assumption of isotropy ('conformal flatness') at the Big Bang or Hawking's proposal of a particular special set of boundary conditions on the wavefunction of the Universe which imply both initial isotropy and homogeneity. He would regard his thesis as satisfied when initial anisotropies (which

* *The Anthropic Principle*, Venice, 18–19 November 1988.

in principle can be estimated from measurements of anisotropies in the cosmic background radiation) are found to be greater than predicted by these special geometries.

This is certainly a controversial viewpoint; apart from the questions it raises philosophically, it needs technical development to be made precise enough

for verification. In fact, it eventually leads to the major unsolved problem of a probability measure on the initial conditions for the Universe, without which all this kind of speculation is on shaky ground. □

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Palaeoclimate

Finer grains of truth

Peter D. Moore

POLLEN analysis of organic peats and lake sediments is used extensively to reconstruct past changes in vegetation and climate. But such studies have concentrated mainly on changes detectable on a timescale of centuries or even millennia. Changes over timescales of a few decades or less are usually considered too fine to be resolved by the technique. But several recent projects show that this is not always so: Green and co-workers^{1,2} for example, not only show that fine-resolution analysis is possible, but also relate it to environmental variables such as short-term climatic fluctuations and fire frequency.

Several problems stand in the way of fine-resolution pollen analysis. In many lakes, sediment is resuspended and re-deposited during periods of turbulence³; it can even be 'focused' into certain parts of the basin⁴. These processes mix materials over a period of years and, although the general trends in the fossil record may be preserved, short-term changes are masked and smoothed. In peat deposits, the main problem is one of pollen migration within the loosely packed upper layers of the growing peat mass. Experiments in which pollen is added to columns of peat suggest that vertical transport with water flow limits the resolution potential of the analysis⁵.

Despite these concerns, some studies both on lake sites and in peat deposits do permit fine-scale resolution. In deep, steep-sided lakes, resuspension may not be significant and sediments can even develop annual laminations, demonstrating the stability of sedimentation. In peats, several studies involving close sampling, even in poorly compacted layers, show a pollen stratification that argues against vertical pollen migration. Van Geel and Middelborg⁶ have now analysed the stratigraphy of freshly formed peat in Carbury Bog, Ireland, which shows strong peaks in pollen from Ericaceae, Gramineae and *Pinus* in the top 10 cm of unconsolidated peat, formed over the past 10 years. If there were significant downwash of pollen through the upper peat layers, these peaks would have been lost.

Green and colleagues have developed techniques of comparing such fine-scale pollen changes with fluctuations in environmental factors in peat¹ and in lake² sites. By looking at the sequential records of pollen, charcoal and (independently obtained) rainfall data as time series, they can cross-correlate the data sets and also allow for lag times between the causative factor and its response in the pollen record. They find that certain pollen taxa, *Eucalyptus*, grasses and Compositae (Tubuliflorae), show a strong positive response to rainfall, and that even small variations in rainfall are reflected in the pollen stratigraphy. These are also correlations with charcoal deposition. *Eucalyptus*, for example, responds positively with a 5–10-year lag phase, suggesting that trees damaged by fire had recovered rather than being destroyed.

But the relationship between pollen and charcoal is complicated by problems of soil erosion and consequent enhanced pollen input following a fire. And the origins of the charcoal itself must be considered: Clark⁷ has examined charcoal transport in relation to particle size and considers that fine particles (such as those found on microscope slides for pollen preparations) are transported over long distances and do not necessarily reflect fires in the immediate catchment. So correlations between pollen and charcoal must take into account the dispersal characteristics of both pollen grains and carbon particles.

These recent studies indicate that the technique of pollen analysis, both of peats and certain types of lake sediments, does have potential in providing fine-resolution information concerning vegetation changes and hence the variation of other environmental factors in time. □

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Daedalus

Diamonds forever

BRITTLE fracture is the ultimate engineering catastrophe. Microscopic cracks lurk in all solid surfaces; they gape slightly under load, and may even extend a little. Sooner or later the component may meet a stress great enough for a crack to run away, like a cloth tearing in two from a nick in one edge, and disaster strikes.

Now Daedalus has the answer. When a microcrack forms or extends, the local chemical bonds are pulled apart, leaving two pure free-radical surfaces. Such reactive faces must combine almost instantly with local air and water. But, says Daedalus, suppose the atmosphere around the crack were not air, but a polymerizable gas such as ethylene? Each radical-covered surface would be a splendid heterogeneous polymerization catalyst: the crack would be instantly bonded with polyethylene.

But polyethylene is not really an ideal 'glue'. Daedalus recalls that methane-hydrogen mixtures can deposit a tenaciously bonded diamond film on surfaces hot enough to decompose the methane to methyl radicals. A fresh crack surface dense with free radicals should do this even in the cold, by direct electron-transfer. A crack formed in a methane-hydrogen atmosphere should be instantly sealed with strong, stiff, inert, non-melting diamond: the finest imaginable engineering glue.

So Daedalus plans to 'crack-proof' industrial structures such as cranes, bridges and pylons, by slowly stressing them up to working load in an atmosphere of methane-hydrogen. As each microcrack in the structure opened up, it would be automatically sealed with diamond. When the structure finally reached its design load, each stressed part would be studded with tiny diamond inclusions: closing all its potentially deadly little cracks, and pre-stressing it perfectly to its service duty. Even structures like airframes, plagued by the growth of fatigue cracks in service, might be 'immunized' against them by the sealing of the sites from which they begin.

And brittle materials like ceramics could at last be tamed. A ceramic turbine blade, gradually loaded in hot methane-hydrogen, would show its true strength at last. Deprived of the surface flaws from which to fail by brittle fracture, it could be loaded almost up to the point of plastic flow. Thus the splendid corrosion resistance and high-temperature performance of ceramics will be made safely accessible to engineers. All the heat-stressed valves, blades, pistons and cams of modern high-power engines, so vulnerable even in metal, will be safe and durable at last. Even existing engines and turbines might be crack-proofed by pumping them up to speed with hot methane-hydrogen.

David Jones

Lethality of 'killer' bee stings

SIR—It has been predicted that Africanized honeybees (*Apis mellifera scutellata*) will displace European honeybees in warm climate areas of the United States after 1989 (ref. 1). In South and Central America, the highly developed colony defences of Africanized honeybees include a predilection for mass attack

lethalities of the two venoms were calculated (as LD₅₀)^{12,13} from experiments in which Swiss mice and C3H/OuJ mice weighing 20–30 g were injected with venom in doses of 1, 2, 4 and 8 mg per kg, with eight animals per dose.

As shown in the table the lethality of the two venoms was not significantly different

Lethality of Africanized and European honeybee venoms in two strains of mice

	LD ₅₀ i.v. (mg per kg)	95% confidence interval	LD ₅₀ i.p. (mg per kg)	95% confidence interval
Swiss mice				
Africanized bee venom	2.8	2.0–4.1	2.8	2.0–4.1
European bee venom	2.8	2.0–4.1	3.8	2.2–6.4
C3H/OuJ mice				
Africanized bee venom	7.1*	5.7–8.8	ND	
European bee venom	4.6*	3.6–5.7	ND	

ND, not done.

* Significantly different, $P < 0.001$.

during which thousands of stings may be inflicted on one individual^{2–3} causing life-threatening toxic reactions^{4–9}. Although attacks of 300–500 stings have been survived without treatment⁴, more than 500 stings are commonly fatal⁸. Strategies for minimizing the severity of reactions to Africanized bee sting attacks require information on the toxicity and quantity of the venom of Africanized honeybees. We report such data and conclude, by comparison with data from European bees, that the mortality associated with Africanized honeybee attacks is primarily the result only of the number of stings.

Worker (forager) honeybees (mainly *Apis mellifera ligustica*) were captured while returning to their hive in Tucson, Arizona. Attacking Africanized honeybees were captured from a colony in Guanacaste, Costa Rica¹⁰ and identified by two independent morphometric determinations (H. Daly, University of California, Berkeley; S.W. Batra, Smithsonian Institution, Washington). Venom, free from contaminants, was obtained from the venom reservoirs by dissection as previously described¹¹, then lyophilized and stored at -20°C .

Analysis of venom pooled from 1,000 bees of each type showed that European bees contained more venom (147 μg dry weight) than Africanized bees (94 μg), which were also smaller in size. The

in Swiss mice challenged intravenously (i.v.) or intraperitoneally (i.p.), whereas Africanized bee venom was significantly less active than European bee venom in C3H/OuJ mice challenged intravenously.

As the venoms from the two types of honeybee are of similar lethality, and as there is less venom in Africanized honeybees, the serious effects of multiple stings from Africanized honeybees seem mainly to be attributed to the multiplicity of stings. Perhaps use of the popular term 'killer bee' to describe the Africanized bee is inappropriate.

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Sexual advantage

SIR—Kelley *et al.*¹ report a 1.43-fold advantage for sexual over asexual reproduction in sweet vernal grass and thus seem to answer the question of "the ubiquity of sex." However, we find several problems in their methodology and analysis of the data.

First, in an attempt to simulate the natural dispersal pattern of the offspring, Kelley *et al.* planted sexual and asexual progeny conforming to the seed dispersal pattern around the parent. This is erroneous for two reasons. (1) Asexual progeny (tillers) are generally conjoint with the parents and do not disperse as widely as seeds. Planting asexual tillers in a pattern conforming to that of seed dispersal will

grossly overestimate their fitness as it relieves them from competition for resources from the parent plant and also provides liberal space for the daughter tillers to sprout. (2) The natural pattern of distribution of the seedlings surviving to the age at which the authors transplanted the 'test' seedlings would be different from that of seed dispersal around the parent. Although the number of progeny is calculated, this is not used to arrive at the pattern of seedling distribution. Even if it had been, the failure to incorporate data on density-dependent germination and seedling survivorship at various distances from the parent would weaken the analysis. We believe that the planting pattern followed is very unnatural, and that the fitness estimates obtained are therefore incorrect.

Second, the relative advantages of sexual and asexual reproduction was compared using the number of inflorescences produced by the respective ramets. It denies logic to suggest that asexually reproducing organisms gain fitness through organs involved in sexual reproduction such as inflorescences and spikelets. A valid method could be to compare the number of tillers produced by asexually reproducing ramets with that of equal-aged seedlings produced from the total spikelets of the sexually reproducing ramets. Clones of purely sexual and asexual types would be needed for such a comparison, but unfortunately they are not found in sweet vernal grass.

Third, the authors attempt to test "spatially varying environment as a selective force favouring sex" by comparing the relative fitness of the sexual over asexual reproduction for a 2-m distance from the parent. We do not think that the 2-m range is sufficient to represent a spatially varying environment; nor are we provided with any index of environmental variation within this 2-m radius around the parent plant.

Finally, the degrees of freedom for parents in Table 1 of ref. 1 should be 24 rather than 25.

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KELLEY *ET AL.* REPLY—Shaanker and Ganeshaiah seem to have misunderstood the purpose of our experiment. We intended to test the short-term advantage of sexual versus asexual reproduction, and not the advantage of seed versus vegetative propagation. Hence, we were interested in measuring the short-term selective force which would act against a mutant converting sexual to asexual seed production (if and when it arose). Both

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sexual and asexual progeny were thus planted in the locations to which seeds would naturally have been dispersed, because our hypothetical mutant presumably would not alter both the mode of reproduction and the mode of dispersal. The transplanted tillers were similar both in size and in age to seedlings which had germinated the previous fall in the field. We acknowledge that our simulation was a first-order approximation of the natural progeny dispersal pattern in the field, and that it did not incorporate data on density-dependent germination and seedling survivorship. As far as we are aware, few data on this point exist. Nevertheless, our previous experiments have shown remarkably little response of adult tillers and seedlings of *Anthoxanthum* to density variation in the field^{2,3}. Further, the relative advantage of sexual progeny in *Anthoxanthum* is not strongly affected by changes in planting density². We suspect, therefore, that our simulation of progeny dispersal is not particularly unnatural.

Shaankar and Ganeshaiah again seem to confuse asexual reproduction with vegetative reproduction when they argue that inflorescence number is an inappropriate measure of fitness. Hypothetically, asexual progeny could produce apomictic seeds, and hence gain fitness through inflorescences and spikelets. (Using the number of vegetative tillers as an estimate of fitness, our data still show a significant advantage for sexual progeny. The relative fitness of sex is 3.34 ± 0.71 when calculated using the number of vegetative tillers at the end of the second year as an estimate of fitness.)

Our experiment was not designed to test the spatially varying environment hypothesis, and difficulties in testing this hypothesis arise because distance from parent is confounded with progeny density. Nevertheless, the choice of 2 m as the maximal distance for planting was entirely appropriate in *Anthoxanthum*, as 99 per cent of all seeds is dispersed within 2.0 m. Hence, although experiments we have done do show a greater advantage for sexual progeny at greater distances (10 m) from the parent⁴, such considerations are not relevant to explaining how a twofold advantage for sexual reproduction could arise, unless rare long-distance dispersal yields a considerable advantage for the sexual progeny, such as in a colonization event. The plant community within which the present experiments were conducted is relatively stable⁵, and hence it is unlikely a significant short-term advantage for sex arises in this *Anthoxanthum* population from long-distance dispersal.

Shaankar and Ganeshaiah are correct to note that the degrees of freedom for parents, distance $\times$ parent, and parent $\times$ sex should be 24, not 25. However, the effect of this error was to deflate the *F*-values. The correct *F*-values are of

higher significance, and our conclusions are unaffected. In summary, we believe our results are sound and provide clear evidence for a short-term (within a generation) advantage for sexually reproducing females.

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Thoroughbred breeding

SIR—Gaffney and Cunningham¹ and Hill² have addressed a biological dilemma with important consequences by illustrating that although TIMEFORM ratings for thoroughbred horses are increasing by about 1 per cent per year, the winning times for the English classic races have not changed appreciably since 1930. I propose that there is insufficient variation in speed realistically to expect significant reductions in winning times, and improvement in TIMEFORM ratings may be due to components of racing ability other than speed.

The genetic principle under investigation is that of a correlated response in winning time to selection based on TIMEFORM ratings. The expected correlated response per generation (CR) can be calculated from the formula³ $CR_s = ih_s h_T r_G \sigma_s$, where *S* represents speed, *T* the TIMEFORM rating, *i* the intensity of selection, *h* the correlation between phenotype and genotype, σ the phenotypic standard deviation, and r_G the genetic correlation between speed and TIMEFORM rating.

One proposition to explain the contradiction is that the standard deviation for speed is very small. Data to compute this statistic directly are unavailable and so σ_s is estimated here from observations of trial races of 1,200 metres (six furlongs). A high proportion of horses trained for racing compete in such trials in which the distance between first and last seldom exceeds 70 metres. A typical winning time for 1,200-metre races is 72 seconds⁴, and therefore a distance range of 70 metres represents a time range of about 4 seconds. Assuming that this range spans 95 per cent or 4 standard deviations of the population the standard deviation for time to run 1,200 metres is 1 second. From Gaffney and Cunningham $(i_m + i_T)/(L_m + L_T) = 0.132$ and $h_T = 0.6$. Substituting these

values in Falconer's formula: $CR_s = 0.132 \times 0.6 \times 1 \times h_s r_G = 0.079 h_s r_G$ seconds per year and taking 0.6 and 0.8 as arbitrary values for h_s and r_G respectively, $CR_s = 0.04$ seconds per year or 2 seconds per half century. This represents a response in speed of 2.7 per cent which is considerably lower than the 5 per cent predicted by Hill, but is still greater than the improvement indicated by Gaffney and Cunningham. But the predicted response will approach zero if the values of h_s and or r_G are lower than assumed above.

Why, then, have TIMEFORM ratings improved by 1 per cent per year whilst winning times have not changed significantly? A possible explanation is that breeders have difficulty in ranking animals across years. In this instance the problem may be compounded by TIMEFORM rating being subjectively assessed and influenced principally by a horse's ability to win races. Although such ratings may accurately rank animals within age group, their reliability as a measurement from which to estimate rates of genetic change should be addressed.

On the other hand, the improvement in TIMEFORM ratings may be a true reflection of genetic progress for ability of horses to win races. This 'ability to win' encompasses many traits such as strength, temperament, speed and desire to win. The fact that winning times are not changing may simply mean that speed has become a relatively unimportant component of 'ability to win' in thoroughbred horses during the past 50 years.

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CUNNINGHAM REPLIES—Eckhardt *et al.*⁵ in their comment on our paper¹ suggest that the record times for the Derby in 1987 and 1988 provide evidence that racehorses are becoming faster. These two times do not invalidate our general point that winning times have been nearly static for more than 50 years. The main conclusion of our study¹ was in fact that genetic improvement in racing ability of the thoroughbred population as a whole has been very close to what selection theory predicts. We made the point that winning times of the best horses in the most prestigious races are not an accurate measure of genetic change in the population as a whole.

The speculations of Eckhardt *et al.* about the reasons for slow genetic improvement in thoroughbreds, although interesting, are not necessary, and are also debatable. Eckhardt *et al.* suggest that racing ability calls on such a wide range of structural and physiological factors, some of which are antagonistic to each other, so that improvement in net effect is more difficult than it is for economic traits in other domestic animals. A high-perform-

ing dairy cow, for example, secreting 3 kg of milk solids per day, makes comparably wide demands on her physiology.

Eckhardt *et al.* argue that genetic variation for running ability in horses may have been depleted by a long evolutionary history during which this was an important survival trait. Again, it could be argued that the evolutionary imperative was to outdistance predators, whereas the modern racing goal is to outdistance other horses. The first may have an intermediate optimum; the latter clearly is unbounded. Our estimate of the heritability of TIMEFORM rating, about 35 per cent, is similar to other estimates for different measures of racing ability, and all indicate substantial additive genetic variance.

How can the different question of static (or nearly static) winning times in classic races be resolved? One answer may be that tracks are now watered in dry years. Another is discussed above by Beatson, who suggests that selection on a subjective criterion like TIMEFORM rating produces a negligible correlated response in racing speed because of the very low variance in the latter trait. The low estimate of variability of racing time which he prov-

ides (CV=1.5 per cent) is supported by a similar value (CV=1.3 per cent) obtained by analysing many time records in greyhounds⁶.

Another reason could be that winners in classic races are close to some physiological limit. Lactic-acid accumulation in blood rapidly reaches critical levels in horses undergoing continuous exertion⁷. This would impose limits in longer races but be less restrictive in shorter ones. There is a suggestion of a more pronounced plateau in performance in winning times in the St Leger (1¼ miles), than in the Oaks, Derby and Belmont (1½ miles), with even less evidence of a plateau in the Kentucky Derby (1¼ miles) and the Preakness (1⅓ miles).

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HLA and insulin-dependent diabetes mellitus

SIR—The contribution of the polymorphic residue at position 57 of the HLA-DQ β chain to autoimmune diabetes (insulin-dependent diabetes mellitus or IDDM) has been discussed recently¹⁻³. Genetic susceptibility to IDDM is positively correlated with a neutral residue (Ala, Val, Ser) and negatively correlated with an Asp at this position. Position 57 is also revealed as a critical residue in the analysis of *Pemphigus vulgaris*⁴ but susceptibility to *P. vulgaris* seems to be a property of the allelic β -chain structure rather than just residue 57. Similarly, for IDDM susceptibility, there are several haplotypes with a neutral residue at 57 that do not confer high susceptibility (for example, DR7) and some with Asp 57 that seem to be disease-associated (for example, DR4 and DRw9 in the Japanese). Another challenge for the simple residue-57 model is how to account for the increased risk of DR3/4 individuals relative to DR3/3 or DR4/4

individuals as they are all non-Asp 57 homozygotes. These observations are most simply explained by assuming that other HLA class II sequences can also confer susceptibility to IDDM. The DR β I allele, for example, seems to contribute to the DR4 association with IDDM^{7,8}. In general, it is specific combinations of class II alleles (haplotypes and genotypes) that are most highly associated with disease.

Notwithstanding these and other exceptions⁹, the data implicating residue 57 as critical in disease susceptibility are striking: this intriguing correlation demands an explanation. One possibility is that the region around position 57 of the DQ β chain encoded by a susceptible allele (for example DQ β 3.2) presents an autoantigen to the T cell or determines the expression of a particular T-cell receptor during thymic maturation^{1,4}. The notion, however, that all allelic DQ β chains that contain

Asp 57 (or non-Asp 57) bind a common islet cell autoantigenic epitope or influence the T-cell receptor repertoire in a similar way seems unlikely given the diversity of Asp 57 (or non-Asp 57) alleles (Table 1). An alternative interpretation is that the residue at position 57 may simply influence the overall conformation of the β -chain and not necessarily interact directly with a putative autoantigen. The functional significance of position 57 is suggested by the conservation of this charge polymorphism in all human class II β -chains (DQ β , DR β I, DR β III, and DP β). In fact, position 57 polymorphism at these other β -chain loci may also be relevant to disease susceptibility^{4,5}. Moreover, the same polymorphic residues are found at position 57 in chimpanzee, gorilla, baboon, rhesus and cebus β -chain alleles (Table 2; Gyllenstein *et al.* unpublished observations). The evolutionary maintenance over 10-20 million years of a balanced polymorphism between Asp 57 and Val, Ala or Ser at this position in all class II β -chains examined so far suggests that β -chains containing Asp 57 and those with Ala, Val, or Ser at position 57 may be structurally and functionally different. Perhaps, selection for the presence of both types maintains this polymorphism¹⁰. Whatever the functional significance of residue 57 polymorphism, the general correlation observed between disease susceptibility and the nature of residue 57 may ultimately reveal more about the structure of class II β -chains than about the specific response to a putative diabetes autoantigen.

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Single-strand RNA

SIR—In their News and Views article, Lamb and Dreyfuss¹ state "All RNA viruses have to go through a dsRNA intermediate in their life cycle". If "ds RNA" designates double-helical RNA, this statement is incorrect, for the replication intermediate of RNA phage Q β is a single-stranded minus strand, and not double-stranded RNA². The same is thought to be true of many of the animal positive-strand RNA viruses, including picornaviruses, togaviruses and coronaviruses³, although

Table 1 HLA-DQ β sequences from residues 52 to 57

DQ β allele	Sequence	Group
DQ β 3.2	—PLGPPA—	Ala, Val, Ser 57
DQ β 2	—LLGLPA—	
DQ β 1.1, 1.7	—PQGRPV—	Asp 57
DQ β 1.2	—PQGRPS—	
DQ β 3.1, 3.3	—PLGPPD—	Asp 57
DQ β 4	—PLGRLD—	
DQ β 1.3, 1.4, 1.5, 1.6	—PQGRPD—	

Table 2 Residue at position 57 in class II β -chains

	DQ β	DR β I	DR β III	DP β
Human	Asp Val, Ala, Ser	Asp Val, Ala, Ser	Asp Val	Asp Ala
Non-human	Asp	Asp	Asp	—
Primates	Ala, Val	Val, Ser	Val, Ser	

Primate species examined include chimpanzee, pygmy chimpanzee, gorilla, rhesus, baboon and cebus.

the data are not clear-cut. There is no evidence that double-stranded RNA is an intermediate in the case of transcription and replication of negative-strand viruses (D. Kolakofsky, personal communication); rather, RNA is complexed to nucleocapsid protein, which probably prevents formation of double-stranded RNA.

Double-stranded RNA is nevertheless formed as a dead-end side product of Q β RNA replication, presumably when there is a failure of the mechanism that separates or keeps apart plus and minus strands during synthesis². In the case of eukaryotic minus-strand viruses it may be a similar rare event that gives rise to a double-stranded RNA that can be taken apart by the modifying/unwinding enzyme of Bass and Weintraub⁴ and give rise to hypermutated RNA⁵.

Were double-stranded RNA an obligatory intermediate of eukaryotic viral RNA replication, one would expect hypermutational events to be frequent, rather than very rare⁵. Maybe it is the avoidance of double-stranded RNA intermediates that enables RNA viruses to circumvent lethal hypermutation in eukaryotic cells.

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Human T-cell receptor expression

SIR—Janeway, in his recent *News and Views* article¹, stated that intestinal epithelial lymphocytes “in both chickens and man are predominantly CD8⁺ T cells with γ/δ receptors”. In our immunohistochemical studies of human intraepithelial lymphocytes, we also find that most intraepithelial lymphocytes express CD8, although approximately 20% of intraepithelial lymphocytes express neither CD4 nor CD8.

Using an antibody against the δ -chain of the T-cell receptor (δ TCS1, T Cell Sciences Inc., Cambridge, Massachusetts) however, we have failed to demonstrate a predominance of δ -chain-positive lymphocytes in normal gut epithelium. We are unable to detect any δ -chain-positive cells in human fetal ileum at 20 weeks' gestation, when intraepithelial lymphocytes expressing CD3 are present². We find that only 2 per cent of intraepithelial lymphocytes in normal adult jejunum, increasing to approximately 33 per cent in jejunum from children with newly diagnosed coeliac disease, express this form of the T-cell receptor. These findings suggest that accumulation of intraepithelial lymphocytes expressing the δ -chain of the T-cell receptor is antigen driven, but that in normal, immunologically stable intestine, the proportion expressing the γ/δ heterodimer is small.

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JANEWAY REPLIES—Spencer and Isaacson's statement that human intestinal epithelial lymphocytes may not be enriched in γ/δ T cells is further evidence of the need to correlate anatomy to structure and function. My statement regarding the presence

of such cells in man¹ was based on results using the same antibody carried out by Bucy in Cooper's laboratory, and reported at the last FASEB conference in Las Vegas, Nevada.

I have since learned that another antibody of similar specificity also fails to reveal dominance of such T cells in gut epithelium in man (Groh *et al.*, personal communication). However, we now have evidence that intestinal epithelial lymphocytes of mice are using a V γ :V δ different from that found in any other tissue, including skin. Thus, one must be sure that the reagent used will react with all T cells bearing γ/δ receptors before such conclusions are validated. It is also possible that the results in the mouse reflect the early age at which such cells are collected. The γ/δ receptors in the gut and skin of the mouse are of a form found only very early in ontogeny in the thymus, and seem to rapidly seed these unique peripheral sites. The decline in such cells cited by Spencer and Isaacson may reflect this ontogenetic history.

However, the data of Spencer and Isaacson, and of Groh *et al.*, could reflect a major interspecies difference. If this last hypothesis is correct, would it be too far-fetched to speculate that the reason humans have spontaneous carcinomas, whereas mice seem to have sarcomas and lymphomas as their main tumour types, is that humans have a defect in the disposition of γ/δ T cells in their epithelia, thus allowing transformed epithelial cells to arise and proliferate sufficiently to invade and cause disease?

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Taxonomic stability

SIR—Crisp and Fogg¹ correctly point out that changes in names for taxonomic reasons, which arise from differing opinions on classifications or new data, are tiresome. Nevertheless, when changes arise from a more thorough understanding of evolutionary relationships they must be accepted as part of the development of science, as stressed by Newman².

The current International Union of Biological Sciences *Lists of Names in Current Use*³ initiative aims to limit changes arising from the application of International Codes^{4,5}, rather than as a result of new scientific research. The most effective tool for restricting irresponsible taxonomic changes is peer pressure. Progress in that direction is starting to be made in the Ascomycotina, the largest class of fungi (6,100 generic names; 29,000 species). An interactive eclectic approach to the production of a generally accepted system for ranks from genus to order was initiated in 1982, based on a twice-yearly publication of this institute and the University of Umeå, *Systema Ascomycetum*, which reports changes in classification and reactions to them. Depending on worldwide peer reaction, changes are incorporated into an annually revised “Outline of the ascomycetes”⁶, providing recommended dispositions of generic and higher ranks. Users have an annually updated listing to which they can refer. Parallel publications could clearly be initiated for other groups of organisms, were the necessary resources made available.

At the species level, the International Commission on the Taxonomy of Fungi (ICTF) produces a series “Name changes in fungi of microbiological, industrial and medical importance”⁷. The reasons for changes proposed in the naming of these fungi are reviewed by an international panel of 11 mycologists, and their views are embodied in this series. Again, this could be adopted on a wider front.

International peer reactions are the most likely prospect for restricting unwelcome changes of the type Crisp and Fogg abhor. In the longer term, improvements in the standards of taxonomic practice, such as the ICTF “Code of Practice for Systematic Mycologists”^{8,9}, are required.

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Breaking resistance

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Superconductors: Conquering Technology's New Frontier. By Randy Simon and Andrew Smith. Plenum: 1988. Pp.326. \$28.74, £16.

To physicists and engineers, the lure of superconductivity lay perhaps in its resemblance to magic. They had been taught that the consequence of passing electrons through wires was heating. Although true in the everyday world, there was nevertheless a wonderland where the usual laws according to Ohm and Ampère did not operate. It was a winter wonderland, a little chamber covered with icicles. The magic effect only took place near absolute zero, requiring an expensive coolant, liquid helium. The first attempt to harness the magic failed; a generation of engineers who hoped to use superconductivity in computers had their dreams frustrated because of the price of cooling. Then, in 1986, materials scientists made the first high-temperature superconducting ceramic systems.

This breakthrough has caused an unusual flutter in the wider world. In part this is because some people believe that superconductor magic has broken out of fairyland and is about to transform our world. There is thus a place for a book which attempts to tell people how superconductivity works and how it might, indeed, change the world. Randy Simon and Andrew Smith tackle this task in straightforward fashion. Their writing is informal but makes no concessions when a difficult point is to be made. If readers have to stretch their imaginations a bit, the authors give them due warning and provide the tools of analogy to help them along. One reason for this confident style is that both authors are physicists who themselves work on high-temperature superconductors for an aerospace firm. They are in the forefront of their field, and have probably had to explain the same points to their managers a hundred times. At the same time, the writing is highly polished. This is no journalistic paste job.

Twelve chapters take the reader logically through the history, physics and engineering of superconductors, and describe both the old and new versions. The old, low-temperature (LT) superconductors are based on alloys of niobium, tin and lead, and are cooled by liquid helium or neon. The new, high-temperature (HT) varieties are based on cuprate ceramic compounds, and operate at temperatures near that of boiling nitrogen (high temperature in this context). These are dealt with in the final four chapters of the book, which discuss the excitement of the advances that have been made

in the past three years. Chapter 13 gives a historical account of the initial discovery in 1986; and Chapter 14 describes the most popular research material, yttrium-barium-copper-oxide.

In the last two chapters the authors consider the future. Here, however, they have not done a real analysis of the uses and economics of the new materials, so their predictions are qualitative. But their views are logical — and agree with those of many reasonable people.

In the 1970s, the goal for the superconducting computer was to make a device, cooled by helium, that would fit inside a teacup and could reel off the names of every person in the world in one second. The efforts failed (the computer equipment looked like a kitchen stove), primarily because of the poor thermal properties of helium. Such computers had some highly specialized uses, including many military applications, but by the early 1980s research in the United States was faltering. The authors do not clearly explain why this was so, but they do spell out with enthusiasm the case that was being made for the continuance of LT research. This case was based on the advantages of the LT superconductor — speed of switching, absence of electrical noise and uniquely precise action in sensor devices.

The authors are confident that the

discovery of HT effects will reverse the trend and revive research into LT superconductors. But they fail to mention a strong argument for continuing LT work, namely that the experience of workers in that technology might be harnessed to explore the possibilities of the HT effect. At the very least, LT research should continue while the basic physics and materials science of the new systems are studied.

We are by no means sure that the HT effect will do the things we want. Higher temperature is bound to erode one of the three advantages mentioned earlier, low-noise operation. Furthermore, the lattice structure of the new materials impairs electronic functions and may present insuperable barriers to widespread commercial success. We can only hope that the speed and precision of many classical superconducting effects are not affected too badly by this materials problem. Nevertheless, it will be surprising if the HT effects do not find some startling, novel uses.

Without trying to be prophetic, the authors set the scene for the growth of HT superconductors into a high-technology industry. Their well-balanced account of a complex technical challenge is likely to help laymen appreciate what is going on; many scientists who need a quick review of the subject will also want to read the book. If zero-resistance materials and new quantum-effect devices are going to come out of the winter wonderland and into the real world, then we all need to know about them. □

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Face of the past — a Colossal Head, in total 2.84m high, fashioned from basalt and typical of the Olmec culture of Mexico (c. 1200–900 BC). The picture is reproduced from the new paperback of Michael D. Coe's *Mexico*, 3rd edn, to be published by Thames & Hudson on 13 February, £6.95.

Question time for psychologists

Stuart Sutherland

Stevens' Handbook of Experimental Psychology, 2nd edn. Vol. 1 *Perception and Motivation*; Vol. 2 *Learning and Cognition*. Edited by Richard C. Atkinson, Richard J. Herrnstein, Gardner Lindzey and R. Duncan Luce. Wiley: 1988. Vol. 1 pp. 905, \$85, £77.50. Vol. 2 pp. 1,027. \$95, £85. Two-volume set \$180, £160.

IN PSYCHOLOGY, old problems never die, they only fade away. On the other hand, new problems emerge at a remarkable rate. So it is instructive to compare the second edition of *Stevens' Handbook of Experimental Psychology* with the first, which was written nearly 30 years earlier.

The 'phi phenomenon', the illusion of movement occurring without anything being seen to move, received a great deal of attention in the first edition, but is not so much as mentioned in the second. As to the new and largely unanswered problems, here are two. First, when an ambiguous word (such as 'bank') is encountered, do people unconsciously access both meanings of it or are they directly guided by the context to the correct interpretation? Second, it has recently been discovered that people are very bad at probabilistic reasoning. For example, they fail to obey Bayes' law since they attach far too little weight to prior probabilities. Why this should be so remains unclear, although it may in part be due to a tendency to access the most salient material. For example, most people wrongly believe there are more words beginning with 'r' than words having 'r' as a second letter, because it is easier to recover from memory words starting with 'r' than words having 'r' in any other position.

Stereopsis

The failure to solve old problems and the constant emergence of new ones suggests that psychology makes little progress, but this is not entirely true. For example, in stereopsis the task of deciding which points on each eye are being stimulated by the same points in space (the correspondence problem) is an ancient one, but there are now plausible algorithms that can execute it. These receive only a cursory mention in the chapter on visual perception: unfortunately some psychologists still regard workers in artificial intelligence as rivals rather than allies.

Although the most secure advances may have occurred in vision and hearing, many discoveries have been made and a

few theories put forward in other areas that are unlikely to be completely swept away. The notion that a concept may be based on a prototypical instance to which other instances bear a family resemblance goes back to Wittgenstein, but more recently it has been experimentally shown to apply to at least some concepts. As Eliot Hearst shows in an exceptionally clear and thorough chapter, even animal learning, currently a neglected field, has taken a dramatic step forward. It has been found that the formation of an association between two stimuli (for example, the conditioned and unconditioned stimuli) depends not merely on how frequently one is followed by the other, but on the second stimulus occurring more frequently after the first than in its absence. From a functional standpoint, this makes good sense. Animals, and people, need to learn about stimuli that predict an event better than other stimuli, not merely about random connections between the members of a pair of stimuli.

In psychology, the discovery of new phenomena has usually been determined by changes in the *Zeitgeist*, which in turn have often been influenced by the importation of new ways of thought from other disciplines. Elderly experimental psychologists, such as myself, have seen the subject successively transformed by cybernetics, information theory, Chomsky's generative grammars and artificial intelligence. All four movements suggest that it may be possible to describe and explain complex information-processing with rigour; hence the swing of experimental psychology away from behaviourism to a cognitive approach. The word 'cognitive' has been broadly interpreted because it includes not merely conscious mental processes but the unconscious ones that underlie them. Oddly enough it is the unconscious processes on which most progress has been made. Whereas there are plausible, but unfinished, theories about object recognition (a topic completely omitted from both editions), pitch perception, auditory localization and speech perception, theorizing on the higher realms of cognition remains vague. We have very little idea how memories are accessed and *a fortiori* how they are organized. And although it seems certain that reasoning proceeds by manipulating the semantic content of mental models rather than by the formal application of a logical calculus, we can hardly begin to specify what these mental models are like.

The influence of the information-processing sciences on psychology is new, but that of neurophysiology and related disciplines goes back many years. Today, hacking out pieces of animals' brains more or less at random has been largely supplanted by the study of neurological patients whose brains have suffered

random damage through strokes, tumours or accidents. Such research is a secure and easy route to a PhD or an enlarged list of papers. But although it may provide information on where in the brain a given function is localized, it rarely yields any evidence that is relevant to the really important question of the nature of the mechanisms that execute it. Again, although Bartley Hoebel provides a detailed and scholarly account of recent work on neurotransmitters, the knowledge that they are heavily implicated in a variety of motivational systems, or even the more specific knowledge that the catecholamines play a role in the 'pleasure centres', does not appear to have advanced our knowledge of the mechanisms underlying reinforcement — let alone pleasure.

Experiments

The *Handbook* is not for beginners. It is written with the accuracy one might expect from the 40 or so distinguished contributors, but many of the chapters will be opaque to psychologists who do not themselves work in the field under review. There is little attempt to place a topic in perspective or to state clearly which problems (if any) have been solved and which remain unsolved, or even to decide what the important issues are. Instead, the reader is too often presented with a mass of experiments unadulterated by theory. Experiments are described that were intended to support theories long since shown to be fallacious — for example, those of A.M. Collins and M.R. Quillian that purported to show that the attributes of classes are held hierarchically, so that in memory yellow would be attached to canary, but flying would be attached to a higher node (bird). With several interesting exceptions, such as the chapter by S. Glucksberg and G.A. Miller on the psychology of language, most of the contributors seem to believe that if an experiment has been conducted it must be worth reporting.

Of course, experimental psychology has always lived up to its name and has tended to place more weight on experiments than on theory. The subject is, however, at present undergoing a fifth revolution, namely the development of simulated neural nets consisting of units randomly connected together, in which learning is represented by changes in the connectivity. Such nets have been found to be capable of performing surprisingly complex tasks and have been hailed as the theoretician's paradise. Unfortunately they are unlikely to introduce theoretical rigour into psychology if only because, for the most part, nobody understands how they work. □

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The view from Leningrad

A. Barrie Pittock

Climate Shocks: Natural and Anthropogenic. By K. Ya. Kondratyev. Wiley: 1988. Pp.296. \$54.95, £50.

IN THE foreword to *Climate Shocks*, Will Kellogg describes the book as "an extraordinary *tour de force*" and "a scholarly achievement that is unmatched, to my knowledge". Unfortunately, I cannot agree. The book is certainly interesting to a relative insider, and it raises a number of points which deserve further attention. But, despite his eminence, Kondratyev puts forward a rather idiosyncratic view which will leave many readers bewildered rather than enlightened.

After a brief introduction to physical climatology, there follows an account of the greenhouse effect, and longer chapters on the climatic consequences of volcanic eruptions and on those that might result from a nuclear war. The latter contains a large section on the possible effects of the Tunguska meteor fall in 1908. A final chapter purports to present recent results and conclusions, but in fact largely summarizes the unrefereed papers that were presented at one particular conference held in 1985.

Throughout the book Kondratyev tends to give rather uncritical, detailed and technical summaries of selected papers, many of which are out of date, there being frequent confusion between the views contained in the original papers and those which the authors now hold. The result is a perplexing array of often contradictory statements, with too little attempt to mould the material into a rounded overview.

A central theme is the author's conviction that oxides of nitrogen, supposedly produced in large quantities by the atmospheric nuclear bomb tests of the 1950s and 1960s, caused a significant surface cooling in the Northern Hemisphere (an 'anti-greenhouse effect'), and that they would have caused a decrease in stratospheric ozone had it not been for a simultaneous injection of water vapour into the stratosphere. Kondratyev invokes the mechanism to reconcile the observed average surface temperature trends in the Northern Hemisphere in the 1930s through to the 1970s with the expected warming due to the greenhouse effect. But this requires him to add a contribution from industrial NO_x production before the bomb tests; as this input is tropospheric, the theory hardly stands up to close examination.

The NO_x theory is based on a controversial paper by Hampson (*Nature*, **250**, 189; 1974) and a time series of NO_x con-

centrations derived by Kondratyev and coworkers by working back from the time series of ozone amounts. On p.177 he blames other people's failure to agree with his interpretation of the ozone record following the nuclear tests on rejection of the Soviet data obtained with the M-83 ozonometer; yet on p.192 he concedes that turbidity caused errors in the M-83 measurements.

Nitrogen oxides are also invoked to help 'explain' phenomena associated with the Tunguska meteorite event. Much space is devoted to explanations of the time series of atmospheric optical thickness over Mt Wilson, which shows peaks

estimates of the mass, velocity and other characteristics of the Tunguska meteorite, which Kondratyev says was a cometary nucleus composed largely of solid methane, seem to be plucked out of the air. Perhaps the primary literature is more convincing.

Kondratyev's account of the climatic consequences of nuclear war is largely built on selected early results, with an added emphasis on the possible effects of NO_x and other gaseous products. Like a number of other reviewers, he fails to identify clearly the critical role of absorption of solar radiation by carbon particles, as distinct from scattering. Use of the terms "optical depth" and "extinction", both of which include scattering as well as absorption, obscures the key point that most scattered light still reaches Earth's surface unless it is absorbed by the carbon particles. Thus on p.197 he quotes Crutzen and Birks as reporting solar radiation "extinction" by a factor of 2 to 150, when in fact they wrote that the "average sunlight penetration to the ground will be reduced by a factor of 2 to 150", which is quite different. This may be a fault in the translation, but it is a common misconception.

Other oddities may also be artefacts of translation — for example, a reference to 1815 as "a year without the sun" instead of "a year without a summer" (p.74).

With regard to the climatic consequences of nuclear war, Kondratyev, like many others, concentrates on the surface-temperature effects, giving too little emphasis to the perhaps more serious and robust results which show that rainfall would be dramatically reduced, especially in the tropics. He also fails to give proper attention to the importance of lofting of smoke due to its absorption of sunlight, and the resulting prolongation of the lifetime of the effects.

There is a natural and useful bias in the book towards the Soviet literature. But there is also a tendency to concentrate too much on results from Kondratyev's own group in Leningrad, and not enough effort is made at least to temper the view from Leningrad by comparing it with relevant non-Soviet studies. The treatment of the global carbon cycle is a case in point, with an extensive reference (p.12) to a controversial paper by Gorshov which claimed that "even after burning all existing fossil fuels, the CO_2 concentration in the atmosphere cannot increase by more than 35% to 40%". No contrary view is given.

In summary this is a confusing and somewhat partisan book, one which is perhaps more interesting for what it says implicitly about Soviet science than for what it contributes to an understanding of its subject matter. □

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IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Perturbation for climate (and much else besides) — Krakatoa blows its top in 1883.

on 4 June 1908 and some 60 days later. The Tunguska event occurred on 30 June, so Kondratyev postulates another "pre-Tunguska stone meteorite" which supposedly disintegrated over Tibet on 4 June, the resulting cloud of particles taking 60 days to travel around the globe twice. Without any supporting evidence for the earlier event, this looks like special pleading. Indeed, the rather detailed

World enough and time

David E. H. Jones

The World of Mathematics: A Small Library of the Literature of Mathematics from A'h-mosé the Scribe to Albert Einstein. Commentaries and notes by James R. Newman. *Tempus Books, 16011 NE 36th Way, Box 97017, Redmond, Washington 98073-9717: 1956/1988. Four volumes, pp.2,448 plus indexes. Distributed by Harper & Row, hbk \$99.95, pbk \$50.*

IN 1940 James R. Newman, a lawyer and writer who had trained as a mathematician, began to assemble a popular historical anthology of mathematical writings. He reckoned it might take him two years. Fifteen years later he delivered the million-word manuscript to his publisher: 133 entries, each with a brief introduction and biography of its author. *The World of Mathematics* finally appeared, in four volumes, in 1956. It was immediately successful — a classic of mathematical popularization. Now Tempus Books has reissued it, still in four volumes but this time in paperback as well as hardback form. How does it read in 1989?

The coverage, eclectic from the first, is beginning in parts to date. The broad historical background of mathematics is still excellent; the sections dealing with mathematics in the physical and social worlds, and with the bases of statistics, stand up for the most part remarkably well. But many of the entries concerned with pure mathematics and its cultural relevance would certainly have been reconsidered by Newman today. The intellectual level remains demanding but not intimidating: appropriate for the enquiring non-mathematician, or the mathematician off-duty. About 20 per cent of the entries are drawn from the research literature, or at least the primary presentation of original work; 30 per cent are educational or polemical pieces; 40 per cent are popularizations of established mathematics; and 10 per cent are stories, or whimsical or discursive essays.

The charm of a good historical anthology is the variety of outlooks and challenges it presents. Here is Francis Galton in 1869 discussing hereditary genius, blandly contravening all the principles of social justice by proving that some people are inherently many times brighter than others. Here is Malthus's famous essay of 1798 on population, condemning most of mankind for most of time to unavoidable misery — and where's the flaw in his argument? Here is Alan Turing in 1950 on whether a machine might think, an amazingly modern essay which still reads cogently after nearly 40 years of massive

developments in computing. Newman was clearly a connoisseur of good literary style. Each of his selections can be read purely for pleasure, even without bothering to follow its argument. Anybody who reads the scientific literature should enjoy the book for this alone; anyone who writes it will probably blush for shame.

The whole huge compilation is far too big to take in at a sitting. It is a book for extended browsing, or a rather eccentric work of reference. I like best the entries dealing with mathematics in the real world. Lanchester on the mathematics of warfare, showing how Nelson's brilliant strategy at Trafalgar closely approached a theoretical optimum; Moseley's account of the X-ray identification of atomic number, probably his last paper before his tragic and pointless death at Gallipoli; Bernard Shaw on gambling and insurance — a departure from his usual concerns, but characteristically spirited and surprisingly sensible; John von Neumann on the workings of the neuron, and the analogy between the brain and the digital computer.

This new edition has not been reprinted. A copy of the 1956 Simon and Schuster publication has been optically scanned and digitally reset (the stupefying intellectual triviality of the tasks that occupy most modern computers would have saddened Turing and von Neumann). The index has been slightly modified; I have spotted just two words of additional text. The publishers have thus missed the opportunity to remedy the most infuriating omission of the first edition: the lack of bibliographical references to the entries. Some can be placed from Newman's commentaries, but many are quite free-floating. It is quite impossible to stand in proper relationship to a piece of writing if you don't know in what context, or crucially *when*, it was written. The annoying detective work needed to pin down each entry from Newman's introduction to it, or from other sources, is almost the only unpleasing feature of this marvellous book. □

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Down to earth

Robert J. Devoy

Quaternary Geology for Scientists and Engineers. By John A. Catt. *Ellis Horwood: 1988. Pp.340. £39.50, \$69.95.*

QUATERNARY geology (or, in a less partisan sense, Quaternary science) has long been the Cinderella of the earth sciences, attracting little attention as a discipline except from small national bands of enthusiasts. It is against this background that John Catt has produced a compact book of seven chapters that goes some way towards helping to redress the imbalance. The text presents information in a simple, practically orientated and narrative style, describing some of the subject's study themes, methodologies and techniques.

In some quarters, the book may not be received unreservedly. It is in places too much a catalogue of dry factual material. The treatment may also be viewed as overly selective and idiosyncratic, a situation which may have arisen from the author's aim of principally satisfying the needs of practical field scientists rather than those of specialists.

Against these criticisms, it should be remembered that non-specialists have to start somewhere. Despite the introductory approach the volume is not intended to be a definitive textbook, and the basic descriptive form will suit such non-specialist readers. Instead of a comprehensive overview, we have rather a helpful collection of working principles and definitions set

within the framework of Quaternary data.

Quaternary science is an interdisciplinary and applied area of research. Its historical origin in nineteenth-century geology, coupled with the subsequent growth of specialisms, has too often led to a view of the subject as preoccupied with stratigraphy, gravels and an arid ordering of glacial-interglacial stages. It is refreshing, therefore, to see included here accounts of relevant specialisms which are clearly rooted in the aims and concerns of Quaternary studies. Geomorphology, soil science and dating techniques are all given chapter status alongside the coverage of basic stratigraphic approaches. A further innovation is the inclusion of chapters on mapping techniques and on the economic and applied importance of the Quaternary studies approach.

Regrettably some relevant areas, such as sea-level studies and palaeoecology, receive minimal and less than up-to-date treatment. Together with deficiencies in referencing the more advanced literature, these lacunae possibly reflect the author's own interests as well as the rapid advances in the literature concerned. Nonetheless the book's strengths outweigh its weaknesses. It contains an innovative and authoritative view of Quaternary science, and will be of great practical value to earth scientists. □

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● Newly published by Unwin Hyman is *Chemical Fundamentals of Geology*, by Robin Gill, intended for geology students and others wanting to get to grips with 'geo-relevant' chemistry.

Penury and potential in Venezuela

Maxine Clarke

Five years ago Venezuela's currency collapsed. Since then the country's researchers have been doing science on a shoestring.

TODAY, 2 February, Venezuela acquires a new president, Carlos Andres Perez, victor of the general election held in December after a year-long campaign. He inherits a stable and, in Latin American terms, remarkably democratic political system. Since the end of the dictatorship in 1958 there has been little organized unrest, and the collapse of the guerrilla movement in the mid-1970s heralded almost ten years of stability. On 'Black



Carlos Perez — unlikely to have a science policy.

Friday' in October 1983, the currency fell from about three bolivars (Bs) to the US dollar to its present thirty-five. Almost overnight, imported goods became impossible to obtain and foreign travel became prohibitively expensive.

Before 1983, oil had propped up the country virtually unaided. But when the oil prices collapsed, escalating interest rates set by international banks crippled the economy. Another 50 per cent devaluation seems to be on the way. In his previous administration (1974–1979), Perez nationalized the oil industry and ended the social unrest of the 1960s by inviting guerrilla leaders to join his government. Hopes that he will repeat the trick for the present crisis were pre-empted by the outgoing president Jaime Lusinchi, who last month announced that Venezuela

will stop paying the principal on about \$20,000 million of its outstanding foreign debt to international banks.

Science inevitably takes a back seat in such economically crisis-ridden times. Perez will probably appoint Jaime Requena as his science minister, but the administration is unlikely to have a science policy. Some Venezuelans think this only realistic given the economic problems. Others believe that investment in research is essential for the future, particularly in developing the nascent aluminium, hydroelectric power and plastics industries.

Although there are many excellent scientists in Venezuela, the government's organization of the research effort is a shambles. Apart from CONICIT, a body

that is Latin American rather than having evolved specifically in Venezuela, there is no organized, national system for awarding grants by scientific merit. Decisions are left up to individual directors of institutes or rectors of universities. Some of them do indeed apply professional criteria in distributing the limited funds available, but the lack of accountability means that others need not be so scrupulous. 'Administrative' costs, for example, can swallow up huge proportions of an institution's budget. Unpopular individuals can fail to have their projects supported, and projects can be supported for political, rather than scientific, reasons. Most researchers who have jobs have them for life, a situation open to abuse. □

The institutes: self-help is the answer

EACH OF Venezuela's research institutes is responsible to the ministry that founded it. IVIC, the institute for scientific investigation, is the largest. Others are IDEA, the institute of advanced studies; INTEVEP, the oil institute; FONAIAP, the agricultural institute; FII, the engineering institute; and CAICET, the Amazon research institute. Most of these do not support much basic research. CAICET, for example, is mainly involved in health programmes.

As in other South American countries, research in Venezuela is administered by CONICIT, which has a budget of some 180 million Bs (about \$5 million) per year. About a third of this is available to scientists who apply for grants, another third goes on students and equipment, and another third on administration. Many scientists are critical of CONICIT for spreading these meagre resources too thinly on new programmes rather than continuing to support existing projects.

Even by its rivals IVIC is said to be the best research institute in Latin America. The problems it faces are typical of the conditions faced by scientists in post-crash Venezuela. It is under the control of the minister of health, who appoints the director, who then can appoint heads of department, distribute the annual budget as he wishes and so on. The staff at IVIC elect a new director every four years, but there have been occasions when the scientists' choice has been ignored — if the candidate belongs to the wrong political party, for example. The government ministers

themselves are often ex-scientists who have decided on a political career in order to implement their ideas about what sort of science should be done, a system that leads to frustration among those researchers who feel that their careers are being directed by people who have some grand scheme in mind, rather than those who will take notice of project applications from the grass-roots level.

This frustration has resulted in fragmentation of resources. Two breakaway institutes have been set up by IVIC scientists who in the past have disagreed with the way the institute is run: INTEVEP, which was formed by members of the chemistry department, and IDEA (see below).

IVIC receives an annual budget from the government of 300 million Bs (about \$8.5 million). There are 12 scientific departments, called centres, service departments and administrative offices. The institute also provides facilities such as accommodation, a kindergarten, a national guard post, a bank and that Venezuelan rarity, a post office.

In the past few years the budget has become inadequate for IVIC to function effectively — the money has to pay for 100 scientists, their support staff, equipment and running costs. So other sources of income are being sought. IVIC's director, the neuroscientist Horacio Vanegas, is involved in discussions with various international science organizations and the Andean Development Corporation (CAF), a biotechnology development organization. Shopping around in this fashion is

These articles are based on a recent visit to Venezuela by Maxine Clarke, Nature's Features Editor. Thanks are due to her host Maria Centeno, and also Carlo Caputo, Nora Castaneda, Stephen Garner-Winship, Erica Jaffe, Raul Padron and Jorge Villegas.

still alien to Venezuelans, many of whom have not come to terms with the fact that since 1983 the country is no longer relatively wealthy. Other Latin American countries, such as Brazil and Costa Rica, have set up links with international funding agencies, but oil-rich Venezuela has not, and suffers from being perceived as prosperous by the outside world.

A typical salary for an established staff scientist is about \$350 a month, and the 100 researchers at IVIC have to share \$1 million a year for running costs, equipment, technicians' salaries and so on. Many of the researchers have been graduate students or postdocs in universities in the United States and in Europe, and some manage to obtain funds using the contacts they have made there. Others have begun collaborative projects, enabling exchange visits. "It's a pity we aren't baseball players", said one researcher, "then there would be plenty of government money available." (Baseball is the national sport, and its teams are conspicuously well-sponsored.)

IVIC provides various courses for undergraduates from the Central University in Caracas, about 150 of whom each year go on to study for higher degrees. When IVIC first began to run PhD courses in the 1970s, the universities refused to recognize the institute's right to award the degree. IVIC's response was a typical piece of Venezuelan pragmatism; it simply renamed the degree PhSc (*philosophus scientius*).

Unofficial responses

The researchers themselves have developed many unofficial responses to the economic crisis in which they now find themselves. Officially, there is a preferential exchange rate for government employees of 14.5 Bs to the US dollar rather than the commercial rate of 35 Bs. But any researcher trying to obtain equipment or materials using this method will experience horrific bureaucratic delays. There are stories of how companies have gone bankrupt between receipt of an order and the payment of the cheque (stopping the process at this point is virtually impossible). There is no efficient way by which customs identifies the goods as different from commercial goods, so degradable chemicals, including isotopes, can be delayed for months. It is not surprising that smuggling is the most common way by which scientists obtain the tools of their trade. One brought in his computer with the help of a brewery, whose beer is the only brand IVIC staff will drink.

Many people have built their own equipment out of the materials they have to hand; jury-rigged modem connections are common, and one researcher had made an optical diffractometer using parts of his own camera. This same person

built a rotating-anode X-ray generator out of parts ordered painstakingly slowly from a British company, and then built an office on the top of the machine.

Vanegas, caught between his frustrated scientists used to better times and an unsympathetic government, emphasizes self-help as the only way forward. For the first time in the institute's 30-year history, he is going to produce a five-year plan to identify potentially fruitful areas of research. He is particularly keen on biotechnology, both plant and medical, as an area to attract funds and one that will reap rewards in the future. He is also proud of his institute's technology centre, which provides an analytical service for which it charges. He believes the service provided cannot be bettered anywhere in Latin America, and now that industries and hospitals are unable for financial reasons to send samples for analysis outside the country, they are beginning to use the IVIC service routinely. The profits are divided equally between the institute, the laboratory that has provided a particular test and the people who perform the test. Last year, the centre made 4.5 million Bs (\$130,000) profit.

But at IVIC as elsewhere in the country, science is done in an eccentric and personal way. Instead of groups of researchers working on related aspects of a problem, such as can be found in any institution in the United States or Europe, individuals work on their own project, acquiring research students and technicians, rather than collaborating with other institutions and making the best use of resources. The physics department at IVIC is an exception to the rule, mainly as a result of an attempt to bring together all Venezuelan experimental physicists into one centre. The ratio of theoretical to experimental physicists at IVIC is about 50/50, compared with 80/20 at the universities and 90/10 among graduate students. Miguel Octavio of the cold-temperature laboratory at IVIC says that only one doctorate in experimental physics will be awarded in Venezuela in the next three years, compared with about 30 theoretical degrees.

Octavio has worked on superconductivity for many years, and has been a wry observer of the explosion of the field since the breakthrough made in 1986. Because of his contacts at MIT, where he used to work, he manages to keep in touch. But to use his undoubted expertise in the field to do research on the high-critical-temperature materials, he needs money, and this is conspicuously lacking.

Despite the lack of any compelling reason to do good science — low salaries, nepotism and the difficulty of obtaining materials and information — many people are doing excellent research. This is most apparent at IVIC, where the laboratories are bustling, busy places. Many have

taken a conscious decision to abstain from the in-fighting for privilege and try to do the best research and teach as many students as possible, in preparation for better times. But with their experience of how the country, and its science, are run, few are optimistic; IVIC has lost one-quarter of its staff to positions abroad in the past five years.

IDEA for change

The institute of advanced studies (IDEA) stemmed from acrimony in the 1960s. The then director of IVIC, Raymundo Villegas, conceived the notion of an institute to study the development of science and the interface between science and the arts. When Villegas became minister of science in 1979, he persuaded the government to set up a new institute, and took with him from IVIC his two brothers, Jorge and Leopoldo, and one or two other scientists.

Before the present director, the philosopher Luis Castro, took over in 1985, there were seven scientists and one researcher from the humanities at IDEA. In the past three years, Castro has appointed six more staff from the humanities: an artist, a philosopher, a political scientist and a cultural anthropologist among them. Castro wants to promote the interdisciplinary approach, and create an environment where art and science can meet. The laboratories at IDEA contain plenty of equipment but a marked absence of people to operate it — unlike laboratories in the universities, hospitals and at IVIC, where there are more people than facilities.

IDEA receives a grant of six million Bs (\$170,000) a year, which Castro says is inadequate on its own to run the institute. When it was founded, at a time when the economy was in a better state, IDEA was intended to be an international conference centre, thus generating income of its own. Castro believes that conferences could still be the saving of the institute, pointing out the financial attraction now that the bolivar is so weak.

He stresses that Venezuela has much to offer scientists around the world: there are many unique natural features waiting to be studied, few regulations to restrict experimental programmes (although the bureaucracy of the government is another matter) and a great deal of scientific talent that is eager to undertake international collaboration. The researchers themselves are more sceptical. The troubled World Archaeology Congress is scheduled to hold its next meeting in Venezuela, at the University of Los Andes. But there is not much grass-roots enthusiasm for hosting an event that has become so politically sensitive. Already the manoeuvring over the organization of the congress has begun, diverting energies that would be better spent on research. □

The universities: looking to local relevance

VENEZUELA'S university system is in imminent danger of collapse. From January to May last year, the system was paralysed by a strike of teachers, other staff and students. No classes were held. The cause was the government's failure to keep its promise, made in 1982, to keep staff salaries index-linked to inflation. The strike ended when the government proposed a compromise budget to be implemented last autumn; although university staffs are now back at work, they fear that the worsening economic situation will again allow the government not to keep its promises.

Yet Venezuela has an excellent tradition of university education, in principle free to all. There are 16 public universities and seven small private universities. Venezuela's 'Ivy League' are five 'autonomous' public universities, of which the most important is the Central University in Caracas (UCV), with 50,000 undergraduates. The others are the universities of Zulia, Los Andes, Caribibo and Oriente.

But free university education does not mean free access; in the absence of government loans or grants for the less well-off, higher education remains only a dream for many. Nonetheless, altogether there are more than 300,000 students in Venezuela.

The remaining public universities, set up in the 1960s, are called 'experimental' universities. They were founded at a time when there was much student activist support for anti-government guerrilla forces. At UCV the government converted all the halls of residence into classrooms, an arrangement that still persists, even though student radicalism is now limited to the vocal. The experimental universities have fewer constitutional rights than the autonomous universities, because each one is under the control of the government minister who set it up.

The universities' dispute was triggered by a request for a special loan for running expenses, which the universities were finding hard to meet. When the request was turned down, the strike began, with staff demanding the promised cost-of-living increases, which have not been provided since 1982. In the past three years the cost of living has increased by more than 40 per cent per year. Once the strike began, the students joined in, demanding an improvement in services such as libraries, transport, food and so on.

Postgraduate studies are in an even worse state than undergraduate courses. At UCV, for example, every faculty has a centre for postgraduate studies, but each one has only very few students. Most of them are studying for masters' degrees; there are only a handful of PhD students. The new rector of UCV, Luis Fuenmayor Toro, is trying to increase the numbers of

masters' and doctoral students from the present two per cent of the total number of students at UCV. He also wants PhD students to be more research-based instead of class-taught, as many of them are at present.

Toro believes that one approach to the seemingly desperate economic problem is to emphasize research that is of direct relevance to the Venezuelan people. Many researchers are aware of the need to form partnerships with international colleagues, and feel that Venezuela in better

students at UCV. Situated well outside the city, the only public transport to civilization from USB is an irregular half-hourly bus.

The science departments concentrate on technical subjects, particularly computing studies, information technology, metal alloys, physical chemistry and food chemistry. Biology was born relatively late here, but the department has learnt to optimize its resources, according to Dr Klaus Jaffe, by taking advantage of Venezuela's wealth of natural diversity. Hence there is a palaeontology group investigating dinosaur and other fossils;

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

A cheerful mural at the Central University in Caracas. But university staff remain dissatisfied.

times has perhaps taken a rather haughty view of the rest of South America, a luxury it can no longer afford.

The government invests about 7,000 million Bs (\$200 million) per year on higher education (this figure includes teachers' training). The universities regard this as inadequate, mainly in view of the inflation of the past few years, which has not been mirrored by a corresponding increase in their budgets. The cabinet decides the annual education budget, which is then approved by congress. Once approved, the money is distributed to all the universities by the National Council of Universities. This is done on the 'night of biting' in which each university tries to get the best share. The autonomous universities generally obtain most of the money, but the experimental universities have another potential source of funds: the minister for education. It has become a tradition that the minister of education in a particular administration creates his own experimental university, with a political appointee as dean.

By far the largest and best of the experimental universities is Simon Bolivar University (USB), which supports about 6,000 undergraduate students and 1,000 graduates. The buildings and grounds are beautifully maintained, and the colonial-style architecture betrays the origins of the campus — first a coffee plantation, then in turn a farm for sugar, horses, flowers and now, the joke goes, students. It was set up 21 years ago in response to the guerrilla activities of the

and ecology, marine biology and parasitology departments (although no molecular biology department, not least because up-to-date equipment would be impossible to buy and maintain). Much of the research done here is published in the international literature.

Jaffe believes that the autonomous universities are too large for effective management, and says that the system at USB for distributing money and choosing officials is at least as free as it is in the autonomous universities. The academic staff of the university suggest three names to the government when a new dean is to be elected, and the government selects one of these names. Money awarded to the university is distributed internally according to strict criteria such as productivity, as well as in start-up grants for new researchers. The university receives about five million Bs (about \$140,000) a year for research. This amount is supplemented by the energy of individual researchers, who obtain grants from overseas, particularly Sweden, Japan, Canada and the United States, as well as getting a piece of the small cake distributed by CONICIT. The foreign research money takes almost any form one cares to imagine, from Swedish 'pocket money' to full-blown grants from the National Science Foundation as part of that organization's US-Latin America research scheme. Jaffe says that this constant searching around for money is yet another handicap for scientists in the developing world struggling to keep up with the international pace. □

Segmental patterns of neuronal development in the chick hindbrain

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Identification of specific neuronal populations and their projections in the developing hindbrain reveals a segmental organization in which pairs of metameric epithelial units cooperate to generate the repeating sequence of cranial branchiomotor nerves. Neurogenesis also follows a two-segment repeat, suggesting parallels with insect pattern formation.

THE remarkable diversity of function produced by the vertebrate CNS is based on regional variations in the identity, arrangement and connectivity of nerve cells. A major aim of neurobiology is to understand how such variations develop. One possibility is that cells are organized into repeating groups, or segments, along the antero-posterior (A-P) axis, allowing each segmental population to individuate from its neighbours. Although segmentation is prominent in the development of many invertebrates¹⁻⁴, its contribution to vertebrate development is less clear cut. During this century at least, only the somitic and nephrogenic mesoderm have been regarded as being properly segmented, but it is possible that segmentation is also an important pattern-forming process within the neural epithelium, where transient periodic swellings, neuromeres, have long been recognized⁵⁻⁷. In higher vertebrates, neuromeres are particularly conspicuous in the developing hindbrain; an extensive literature has described these 'rhombomeres' in the embryos of many species, amongst which the numbers and arrangement of swellings are constant (reviewed in refs 8, 9). The original descriptions of rhombomeres culminated in two opposing views of their significance, clearly outlined in 1918 by Neal⁷: first, 'the non-phylogenetic interpretation that neuromeres are either a) artifacts due to the action of fixing agents or, b) transient embryonic structures resulting either from longitudinal compression of the neural tube or to the local strain of related nerves'; and second, 'the phylogenetic interpretation that neuromeres are the visible remnants of a primitive segmentation of the nervous system and consequently reliable clues to the original number of metameres in the vertebrate head'. There have been surprisingly few attempts to relate this gross pattern of swellings to any underlying patterns of neuronal development^{7,10,11}. Some authors did suggest that there may be a correlation between the development of particular cranial nerve nuclei and their origins from individual rhombomeres^{8,10,11}, but others disputed such relationships⁷. This uncertainty was probably a result of limitations in the techniques available at the time. An understanding of the significance of rhombomeres will require the elucidation of their cellular basis. This is also necessary for the interpretation of the expression patterns of genes with putative regulatory roles during the regionalization of higher vertebrates. Many mouse homeobox genes, for example, have boundaries of expression that lie within the developing hindbrain¹²⁻¹⁷.

Here we show that the neuronal pattern of the chick hindbrain does develop on a segmental basis, and that the early arrangement of the cranial nerve nuclei reflects these segmental origins. We find, moreover, that the pattern of hindbrain segmentation shares certain features with the segmentation process described in organisms such as *Drosophila melanogaster*.

Segmental patterns

Rhombomeres arise between stages 9 and 12 and persist up to stage 24 (ref. 9; Fig. 1a). When the full complement of rhombomeres has been generated, the neural epithelium is subdivided

into repeating units by an iterative pattern of neurogenesis. The first neurons appear within alternate rhombomeres. At stages 13-14, axons of these reticular neurons are most numerous in rhombomeres 2, 4 and 6 (r2, r4, r6), whereas neurofilament immunoreactivity in r1, r3 and r5 is mostly confined to cell bodies of newly-differentiated neurons (Fig. 1b, c). At stage 16 the alternating density of neurofilament staining is maintained by the growth of motor axons within the even-numbered rhombomeres (Fig. 1d, e). Between stages 16 and 17 this alternation diminishes as motor axons also begin to appear in the odd-numbered rhombomeres (Fig. 1f).

Rhombomere boundaries

Transversely oriented boundaries between adjacent rhombomeres are revealed by concentrations of axons visible in neurofilament-stained preparations from as early as stage 13 (Figs 1b, 2a). In the boundaries, large numbers of neurones differentiate beneath the segmental ridges of the ventricular surface.

As medially-directed axons approach the midline floor plate, many turn into the medial longitudinal pathway, contributing projections to other CNS regions. Such axons are particularly concentrated at the r4/5 and r5/6 boundaries (Fig. 1b-e). Other axons continue to grow across the floor plate before turning into the contralateral medial longitudinal pathway (Fig. 1e).

At the boundaries, neuronal cell bodies and axons populate embrasures underlying the ventricular ridges (Fig. 2b). The intercellular spaces in these regions are large compared with those in the adjacent neural epithelium (Fig. 2c, d). Moreover, they are distinguished by speckled immunoreactivity to the extracellular matrix glycoprotein laminin (Fig. 2e). Expression of the highly sialylated neural cell adhesion molecule N-CAM in the ventricular layer, in contrast, is localized to the central regions of rhombomeres (Fig. 2f). Finally, Ng-CAM/L1 is expressed at the rhombomere boundaries (Fig. 2g).

The boundaries between rhombomeres are therefore distinct from the central regions; the large intercellular spaces may permit axons to extend preferentially, thereby forming discrete segmented pathways across the transverse axis. The local expression of laminin suggests another way in which this process may be facilitated and Ng-CAM/L1 expression suggests that some axons fasciculate when growing in these transverse pathways.

Cranial nerve roots

The exit points of the cranial nerves occupy distinct positions in relation to individual rhombomeres (Figs 1c, f, 4). The Vth (trigeminal) nerve root, for example, lies in r2 and the VIIth (facial) nerve root lies in r4. In each case, the root lies in the central furrow of the rhombomere, between the two boundaries. Injection of the fluorescent tracer DiI into the Vth, VIIth and IXth (glossopharyngeal) roots reveals a tight correspondence between the position of each branchial motor nucleus and the

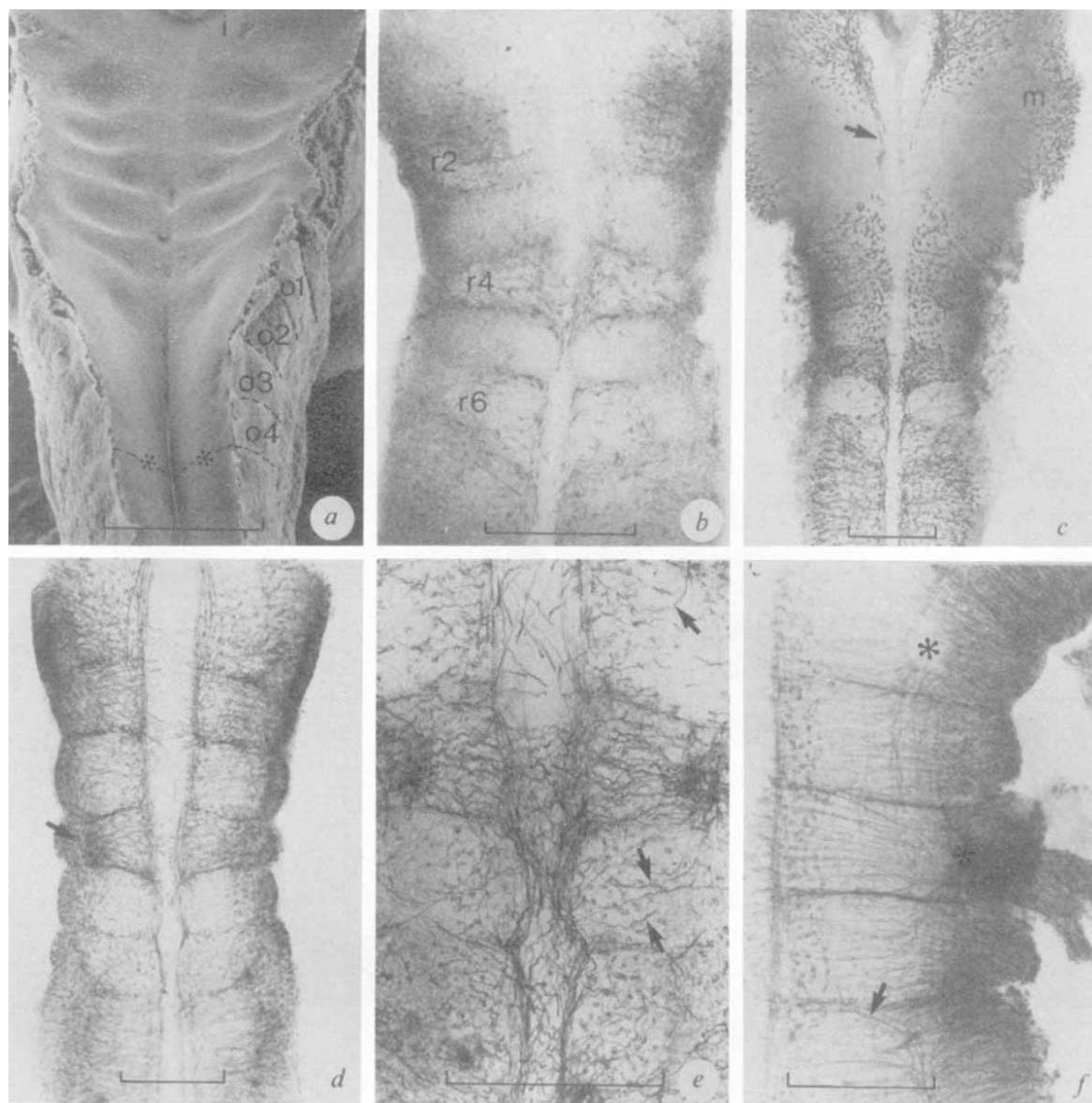


Fig. 1 *a*, Scanning electron micrograph of the chick embryo hindbrain at HH stage 17 (ref. 33). The ventricular (inner) surface of the hindbrain is exposed by removal of the roof plate. A smooth sinusoidal pattern of transverse ridges and furrows is visible along the A-P axis. The ridges do not extend into the midline floor plate. Between the midbrain/hindbrain boundary (isthmus, *i*) and the hindbrain/spinal cord boundary (*) there are six ridges alternating with seven furrows on each side of the midline. Rhombomeres 2-6 each comprise a furrow flanked by two half-ridges. R7 and r8 lie alongside the four occipital somites (*o1-o4*). Before stage 15, a ridge separates these two last rhombomeres at the level of the cleft between the first two somites⁹. Scale bar, 300 μ m. Fig. 1*b-f*, Whole-mounted neurofilament-stained brainstem preparations dissected from chick embryos of stages 13-17, viewed from the pial (outer) aspect. Scale bars, 300 μ m. *b*, Stage 13. Rhombomeres 2, 4 and 6 are indicated, and are distinguished from r1, r3, r5 and r7 by their greater neurofilament immunoreactivity of reticular neurons; axons are also prominent at boundaries. Some axons turn in a posterior direction at the margin of the floor plate. *c*, Stage 14. Neurons have now differentiated in r1, r3, r5 and r7, whereas many axons have appeared in the even-numbered rhombomeres. The midbrain-hindbrain boundary is seen as a sharp change in density of neurofilament staining. In the midbrain, axons are descending from the diencephalic nuclei of the medial longitudinal fasciculus (*mlf*, arrowed). Neurons in the mesencephalic tectum (*m*) are extending axons. *d*, Stage 16. The boundaries between rhombomeres contain clusters of axons, particularly at r1/2, r2/3, r3/4 and r4/5. The root of the VIIth nerve (arrowed) is visible in r4; other roots have been avulsed. The medial longitudinal pathway extends along the length of the hindbrain, adjacent to the floor plate. Commissural reticular axons enter the floor plate from the medial ends of rhombomere boundaries. *e*, Stage 16⁺. The exit point of the VIIth nerve is visible in r4, which is packed with motor axons (compare with Fig. 3*b*). Motor axons (arrowed) are also beginning to develop in r3 (Vth nerve) and r5 (VIIth nerve). Many boundary axons lie outside this focal plane. *f*, Stage 17. Motor axons course laterally in r2 and r4 to reach the exit points (*) of the Vth and VIIth nerves. Axons of these nerves are also visible in r3 and r5, and describe an arcuate, anteriorly-directed trajectory. Axons of the IXth nerve (arrowed) originate posterior to the r5/6 boundary.

Methods. For whole-mount neurofilament staining, embryos were immersion-fixed for 24 hours in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2 at 4 °C. Endogenous peroxidase was blocked by immersion in phosphate-buffered saline (PBS) +0.05% hydrogen peroxide overnight at 4 °C, followed by incubation in anti-68K neurofilament antibody (gift of Dr P. Hollenbeck) +1.0% Triton X-100 for 4 days at 4 °C. Embryos were then incubated overnight in peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham), followed by immersion in diaminobenzidine (DAB, Dotite, 500 μ g in 1 ml Tris pH 7.3) for 3 hours at 4 °C in the dark. Hydrogen peroxide was then added and the DAB reaction product developed at room temperature. The hindbrains were then dissected from the embryos, freed of pial cells, and cut down the dorsal midline. They were then splayed out like kippers to present the entire pial surface, and mounted, without flattening, in cavity slides in 90% glycerol + 10% PBS + 0.02% sodium azide.

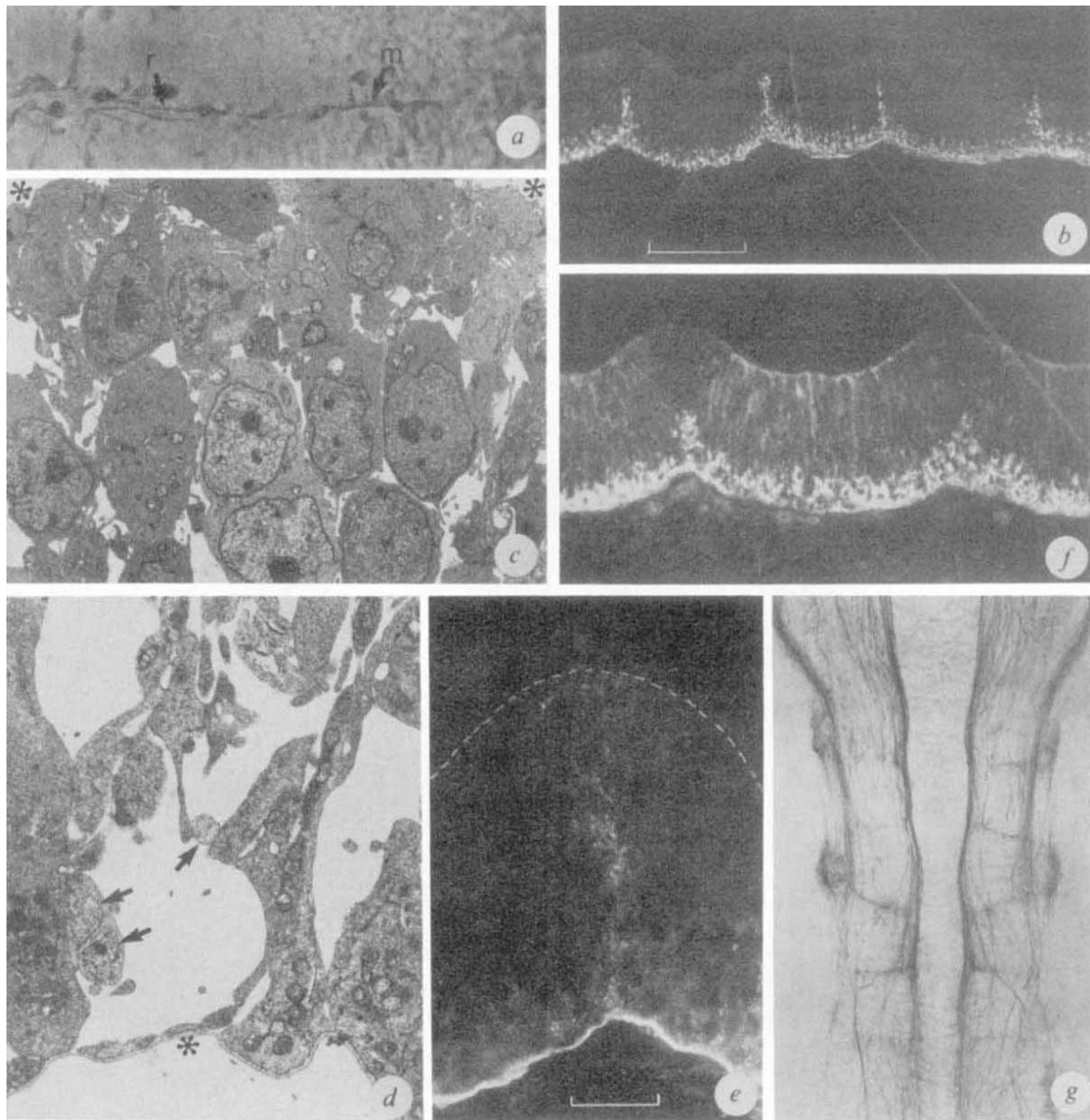


Fig. 2 *a*, Whole-mount stage-16 preparation prepared as described in the Fig. 1 legend, showing reticular (*r*) and motor (*m*) neurons lying at a rhombomere boundary. Medial is to the right. The focal plane is just beneath the ventricular surface. *b*, Parasagittal section of stage-17 hindbrain, stained by the indirect immunofluorescence method using anti-68K neurofilament antibody. At the boundary regions, the mantle layer extends towards the ventricular surface in a delta-shaped embrasure. These dorso-ventral bands of neurofilament immunoreactivity underlie the ventricular ridges. Scale bar, 100 μ m. *c, d*, Parasagittal transmission electron micrograph of a stage 16 boundary region, showing the ventricular surface (*; *c*, $\times 1,890$) and the pial surface (*; *d*, $\times 5040$). The intercellular spaces are distinctly larger near the pial surface and contain several axon profiles (arrowed). *e*, Parasagittal section of stage 17 hindbrain, stained by the biotin-streptavidin amplified indirect immunofluorescence method, using affinity purified anti-laminin antibody³⁴ (JW1, gift of Dr J. Winter). A punctate distribution of laminin immunoreactivity is localized to the boundary region. The speckled immunofluorescence extends throughout the medio-lateral extent of each rhombomere boundary. The ventricular surface is indicated. Scale bar, 25 μ m. *f*, Parasagittal section of stage 17 hindbrain, stained by the indirect immunofluorescence method, using monoclonal anti-N-CAM antibody (5A5, directed against highly sialated form, gift of Dr T. Jessell). Immunoreactivity in the ventricular layer is localised to the central furrows of rhombomeres. Neurons also express N-CAM. *g*, Whole-mount stage 17 hindbrain, stained by the modified indirect immunoperoxidase method described in the Fig. 2 legend, using monoclonal anti-Ng-CAM/L1 antibody (8D9, ref. 35 gift of Dr V. Lemmon). Axons express Ng-CAM in both longitudinal pathways and the transverse boundary pathways.

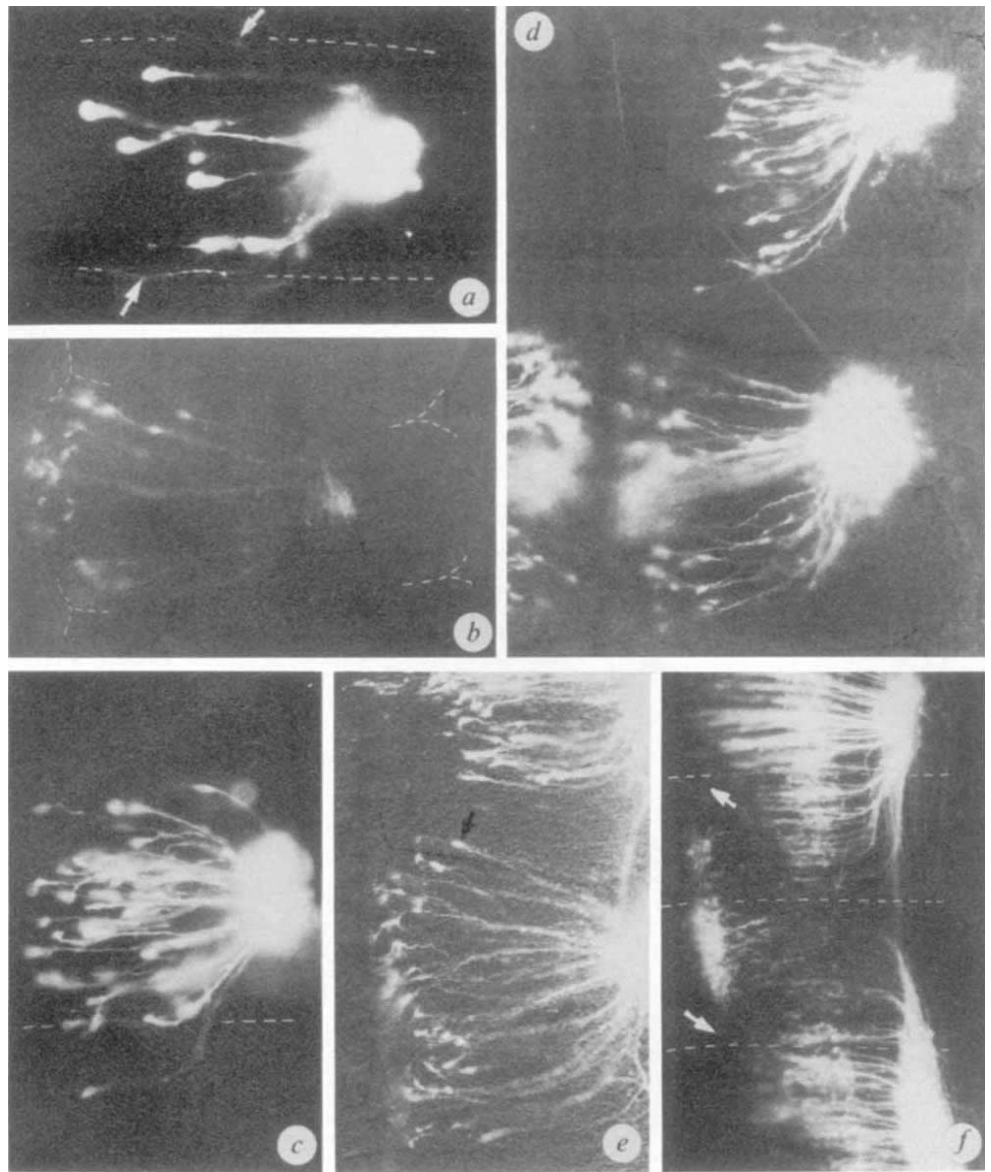
rhombomeric pattern. In a series of injections into 90 embryos, we examined how this correspondence evolves between stage 16, when motor neurons first become traceable by injections outside the brain, and stage 24, when motor neurogenesis and migration are complete.

At stage 16, motor cell bodies of the Vth nerve lie exclusively within r2 (Fig. 3*a*), those of the VIIth in r4 (Fig. 3*b*) and those of the IXth in r6. In each case, the neuronal group spans the A-P length of the rhombomere, and the axons converge towards

their exit point as they course laterally in the marginal layer.

At stage 17 the number of retrogradely-labelled neurons in r2, r4 and r6 has increased, and neurons originating from the alternate rhombomeres (r3, r5, r7) are also labelled (Fig. 3*c, d*). In the case of each branchiomotor nerve, therefore, the first axons arise from the rhombomere containing the nerve root, whereas later axons also arise from the posteriorly adjacent rhombomere (see also Fig. 3*e*). The pathway of motor axons in the odd-numbered rhombomeres is different from that in even-

Fig. 3 Fluorescence micrographs of stage 16–19 living whole-mounted hindbrains after injection of cranial nerve roots with DiI. Anterior is to the top, lateral to the right. *a*, Stage 16. Motor neurons of the Vth nerve lie exclusively within r2. The positions of rhombomere boundaries are indicated by dashed lines; axons (arrowed) lie in both the r1/2 and r2/3 boundaries. *b*, Stage 16. Motor neurons of the VIIth nerve lie exclusively within r4. Axons lie in both the r3/4 and r4/5 boundaries; rhombomere boundaries and floor plate–basal plate boundaries are indicated by dashed lines (combined Nomarski/fluorescence optics). *c*, Stage 16+. Motor neurons of the Vth nerve are more numerous in r2, and a single neuron is labelled in r3. The r2/3 boundary is indicated. *d*, Stage 17. For both Vth and VIIth nerves, neurons are labelled in two rhombomeres. The r1/2, r2/3, r3/4, r4/5 and r5/6 boundaries are indicated at the lateral edge of the neural epithelium (combined bright field/fluorescence optics). *e*, Stage 17. A single motor neuron (arrowed), labelled by injection of the VIIth root, lies anterior to the r3/4 boundary as indicated (combined Nomarski/fluorescence optics). Such exceptions to the general pattern of motor nuclear origins were observed in nine of 34 stage 17 embryos. *f*, Stage 19. Motor axons of VIth, VIIth and IXth nerves originate from distinct mediolateral as well as A–P positions. The VIth nerve nucleus (labelled by DiO, anterior and posterior limits of DiO labelling indicated by arrows) lies medial to, and is co-extensive with, the r5 component of VII and the r6 component of IX (both labelled with DiI). Sensory axons are labelled in the lateral longitudinal pathway. Boundaries r4/5, r5/6 and r6/7 are indicated.



Methods. The cranial nerve roots were exposed by dissection of the pharyngeal roof and the fluorescent carbocyanine dyes DiI or DiO³⁶ (Molecular Probes Inc., Junction City, Oregon, 3 mg DiI or DiO in 500 μ l dimethyl formamide and 500 μ l absolute ethanol) were pressure injected through a 5 μ m tip glass micropipette. Embryos were then incubated for 3–4 hours in oxygenated Earle's balanced salt solution at 32 °C in a 95% O₂, 5% CO₂ atmosphere. The hindbrains were dissected from the embryos and viewed immediately under green (DiI) or blue (DiI + DiO) excitation.

numbered rhombomeres; after an early transverse trajectory, axons turn and cross their anteriorly-adjacent boundary to reach their exit point (Fig. 3*d*). Thus, motor axons cross certain boundaries (r2/3 and r4/5), but do not cross others (r1/2, r3/4 and r5/6). All of the boundaries are crossed, however, by axons descending from the midbrain in the lateral longitudinal pathway and by the ascending and descending axons of cranial sensory ganglion neurons. By stage 19, the Vth, VIIth and IXth nerves are related to a column of motor neurons, extending from the r1/2 boundary to a level opposite the first somite, in which the serially-adjacent branchial motor nuclei are segregated at the alternate rhombomere boundaries r3/4 and r5/6 (Fig. 4).

Injection of DiI into the common root of the vagus (X) and accessory (XI) nerves labels cell bodies in a region that is bordered anteriorly by the r6/7 boundary. Rhombomere 7, therefore, contributes neurons to both the IXth and Xth/XIth nerves.

Although the Vth, VIIth and IXth nerve nuclei occupy serially adjacent positions along the A–P axis, the nuclei of the somatic motor system are discontinuous. The generation of these cells conforms, nevertheless, to a segmental pattern (Fig. 4). The IVth nerve nucleus (trochlear) appears at stage 17 in r1, the VIth nerve nucleus (abducens) lies in both r5 and r6 (Fig. 3*f*) and the XIIth nerve nucleus (hypoglossal) lies in r8. It is interesting that the rhombomere pairing of the VIth nerve is out of register by one segmental unit with that of the neighbouring branchial motor nuclei.

Rhombomeres and branchial arches

The branchial nerves V, VII and IX enter the anterior part of their respective branchial arches (first, second and third). In each case, the arch lies ventral to the pair of rhombomeres in which the corresponding motor neuron cell bodies are located; the clefts between arches 1/2 and 2/3 align with the rhombomere

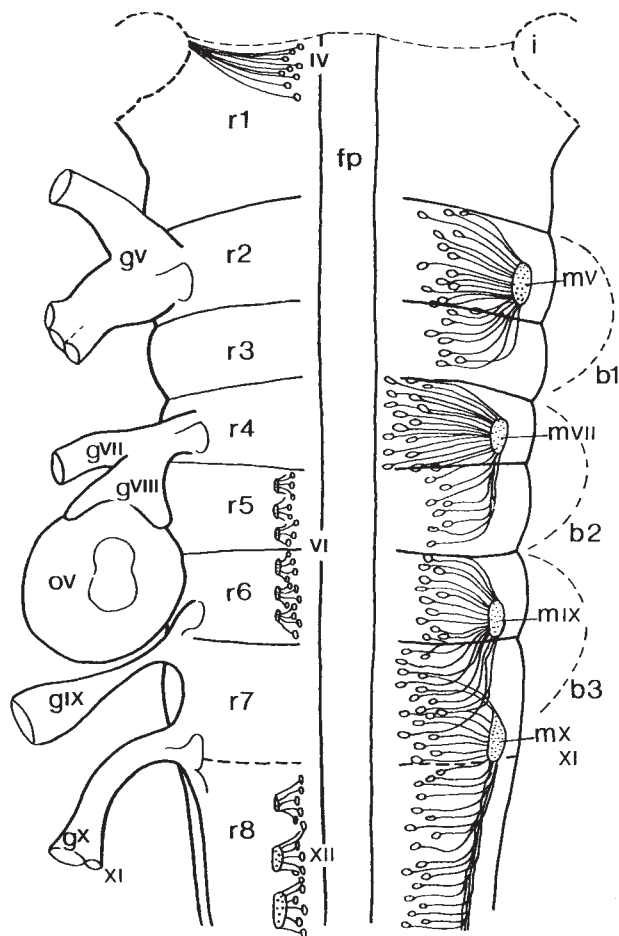


Fig. 4 Schematic diagram of the stage 21 chick embryo hindbrain, based on neurofilament-stained and tracer-injected preparations. The relationship of cranial sensory ganglia (gV-gX), branchial motor nuclei, somatic motor nuclei (IV-XII) and the combined roots of the sensory and branchial motor nerves (mV-mXI) to the rhombomeres (r1-r8) and branchial arches (b1-b3) is shown. ov, otic vesicle; fp, floor plate; i, isthmus/midbrain-hindbrain boundary.

boundaries r3/4 and r5/6 respectively. Thus each of the three principal branchial arches lies in register with the hindbrain segments, in a 1:2 ratio (Fig. 4).

Two-segment repeats

The existence of early segmented neuronal populations and segment boundary-related axons shows that the gross morphological pattern of hindbrain segments finds expression at the cellular level. The process of neurogenesis undergoes a spatiotemporal alternation in successive rhombomeres, involving both the reticular formation and the branchial motor system. Moreover, each branchial motor nucleus occupies a distinct

position with respect to the rhombomere boundaries, and is constituted by neurons from two adjacent rhombomeres lying in register with the appropriate branchial arch. These patterns suggest that there are segment specification mechanisms involving two-segment repetition, as has been described for *Drosophila* segmentation¹⁸. With this in mind, it may be significant that a 'pair-rule'-like pattern of gene expression in the mouse embryo correlates with the alternating pattern of cellular development described here. In E9.5 embryos, the zinc-finger *Krox-20* is expressed in r3 and r5, in each case precisely within the morphological confines of the rhombomere¹⁹.

Segmentation of the neuraxis

The nerve-cord of amphioxus²⁰, and the hindbrain and spinal cord of fish embryos²¹⁻²⁵ contain segmental arrangements of neurons, but these do not exist in the spinal cords of higher vertebrate embryos²⁶. Vertebrate evolution has presumably involved a transition from a segmentally-organised neuromuscular control system for swimming to one more dependent upon long-range integration and control by higher centres. With the attendant loss of intrinsic spinal cord segmentation, the mesoderm has assumed total dominance in trunk segmentation—of skeletal, muscular and peripheral nervous systems²⁷⁻²⁹.

The heads of higher vertebrates, however, retain units of construction, the branchial arches, whose derivatives are both anatomically and functionally distinct. Because these require independent as well as integrated control, segmental motor and sensory units are highly conserved. In contrast to the trunk, paraxial mesoderm segmentation in the head³⁰ is inconsequential, and the lateral plate mesoderm is also unimportant in the patterning process. Rather, it is the neural crest, with its rhombomeric origins, which is responsible for patterning the skeletal and muscular components of each branchial arch^{31,32}. Construction of the head may therefore depend fundamentally on the early segmentation of the cranial neuroectoderm.

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Recombination occurs during telomere formation in yeast

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Short stretches of cloned telomeric sequences are necessary and sufficient for telomere formation in yeast as long as the sequences are present in the same orientation as they are found in vivo. During telomere formation, DNA termini usually undergo RAD52-independent recombination with other DNA termini as would be predicted by models of recombination-mediated telomere replication.

TELOMERES, the physical ends of eukaryotic chromosomes, are essential for the stable maintenance and complete duplication of linear chromosomes. The molecular ends of chromosomes from single-celled organisms to humans bear simple repeats with a defined orientation (C-A rich strand running 5' to 3' from the end towards the interior of the DNA molecule), most of which fit the consensus $C_{1-8}(A/T)_{1-4}$ (refs 1-3). The CA-rich strand usually contains nicks and the GT-rich strand extends into a unique terminal structure¹. Telomere function is also conserved: natural DNA termini from the ciliated protozoans *Tetrahymena* and *Oxytricha* (which bear, respectively, C_4A_2 and C_4A_4 repeats) support the stable maintenance of linear plasmids in *Saccharomyces cerevisiae*⁴⁻⁶. In so doing, the ciliate termini are modified by addition of yeast telomeric $C_{1-3}A$ repeats⁶⁻⁸.

The mechanism for replication of eukaryotic chromosomal termini is unknown. Recombination-dependent replication models⁹⁻¹⁴ (Fig. 1) are attractive because they can account for a number of unusual properties of telomeres, such as their ability to grow and shrink¹⁵⁻¹⁸ and the presence of terminal repeats and single-strand nicks, both of which would promote telomere-telomere recombination. But it is difficult to obtain support for recombination-dependent telomere replication because normally there would be no detectable consequence of terminus-terminus recombination because it involves the exchange of identical sequences located on all telomeres. An alternative model, non-templated DNA replication, became appealing with the discovery of telomerase in *Tetrahymena*¹⁹, a ribonucleoprotein capable of extending the 3' end of the GT-rich strand of different telomeric sequences by the addition of G_4T_2 repeats. The modification of ciliate DNA termini in yeast may also occur via such an activity.

We have determined the DNA requirements for formation of new telomeres in yeast. Our results indicate that 28 base pairs (bp) of cloned telomeric repeats, in the natural orientation and in the absence of any special terminal structure or additional sequences, are sufficient to act as a substrate for the addition of yeast $C_{1-3}A$ repeats. We have also been able to detect recombination between DNA termini, a result consistent with models of recombination-mediated telomere replication.

Cloned telomeric repeats

Linear plasmids were constructed *in vitro* and introduced into yeast by transformation. Each of the *in vitro* constructed plasmids is chimaeric in the sense that its two ends are different. The test end, whose ability to support telomere formation is assayed, carries cloned C_4A_4 repeats, the length and orientation of which are varied in individual experiments. Linear plasmids are named according to the number and orientation of test end

sequences, for example YLp112CA contains 112 bp of test end sequences oriented such that the C_4A_4 -strand proceeds 5' → 3' from the end to the interior of the plasmid, and YLp28GT contains 28 bp of test-end sequences oriented such that the G_4T_4 -strand proceeds 5' → 3' from the end to the interior of the plasmid. The other end of the chimaeric plasmids, referred to as the control end, is a terminal restriction fragment from the extrachromosomal ribosomal DNA of *Tetrahymena* (Fig. 2b) that carries ~300 bp of C_4A_2 repeats and efficiently promotes telomere formation in yeast^{4,5}. Because the *Tetrahymena* fragments are derived from native rDNA, they, unlike the test end, have all of the *in vivo* modifications, such as terminal foldback structures and single-strand interruptions.

Our data lead to a number of conclusions about the molecular requirements for telomere formation (Table 1). First, a test end consisting of 112 bp of telomeric sequences oriented such that the CA-rich strand proceeds 5' → 3' from the end of the molecule, is sufficient to allow the maintenance of a plasmid as a linear DNA molecule in yeast. This result is also observed when the test end carries as few as 28 bp of telomeric sequence although the percentage of transformants containing linear plasmids decreases as the length of the tract of telomeric repeats on the test end gets shorter. In contrast, test termini with 16, 10, 4 or 0 bp of telomeric sequences or termini with repeats in the wrong orientation do not support telomere formation at detectable frequencies.

'Transfer' of terminal sequences

The structure of many linear plasmids of the correct size obtained with constructs containing 28 to 112 bp of test-end sequences was examined by restriction enzyme digestion and Southern hybridization using probes specific for both plasmid termini. For example, DNA from five independent yeast transformants produced after transformation with YLp112CA was digested with *PvuII* which cuts once in the vector portion of the plasmid to produce two fragments (Fig. 3a), a 2.9-kilobase (kb) fragment containing the test end, and a 7.2-kb fragment containing the control end (before addition of $C_{1-3}A$ sequences at each end). One Southern blot of the digested DNAs was probed with $(G_4T_4)_{4.5}$ which detects test-end sequences (Fig. 3b). As predicted, DNA from transformants containing linear plasmids (lanes 2-6) displayed hybridization to a heterogeneous band with an average size of 3.2 kb. The test end probe displayed no cross-hybridization with either yeast or vector sequences (lane 1), or with cloned C_4A_2 repeats (lane 7). The same digestion mixtures run on an identical gel were hybridized to the $(G_4T_2)_7$ probe, which detects the C_4A_2 repeats at the control end of the plasmid (Fig. 3c). DNA from all transformants showed the expected band of hybridization at 8.3 kb. Again, no cross-hybridization between the $(G_4T_2)_7$ probe and either yeast or vector sequences (lane 1), or cloned C_4A_4 repeats (lane 8) was

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Fig. 1 Recombination model for telomere replication (redrawn from ref. 14). Each strand of a DNA duplex is represented by a line: thin lines indicate the GT-rich strand, thick lines indicate the CA-rich strand, dashed lines indicate newly replicated DNA. The 3' end of each strand is indicated by an arrowhead. Nicks in CA-rich strands are indicated by horizontal arrows. Step 1: after DNA replication and subsequent removal of the RNA primer, a 5' terminal gap is produced on each duplex. (For simplicity, only one such gap on a pair of sister chromatids is shown.) Specific nicks are made on the newly-replicated CA-rich strand. Step 2: the free 3' tail (thin line) invades the sister duplex, base-pairs in an out-of-register configuration with the complementary strand (thick line), displacing its complementary strand (dashed line). Step 3: dissociation of the newly base-paired region is facilitated by nicks present on the CA-rich strand of the donor (thick line). Step 4: the resulting internal gap and terminal 3' gap on each duplex is filled by repair synthesis (dashed line) and remaining nicks are sealed by DNA ligase. Replication by this mechanism results in two fully replicated duplex molecules, one of which (duplex on the right) is longer (thereby providing a molecular explanation for how telomeres can increase in length) and has sequence information obtained from the thick duplex on the left.

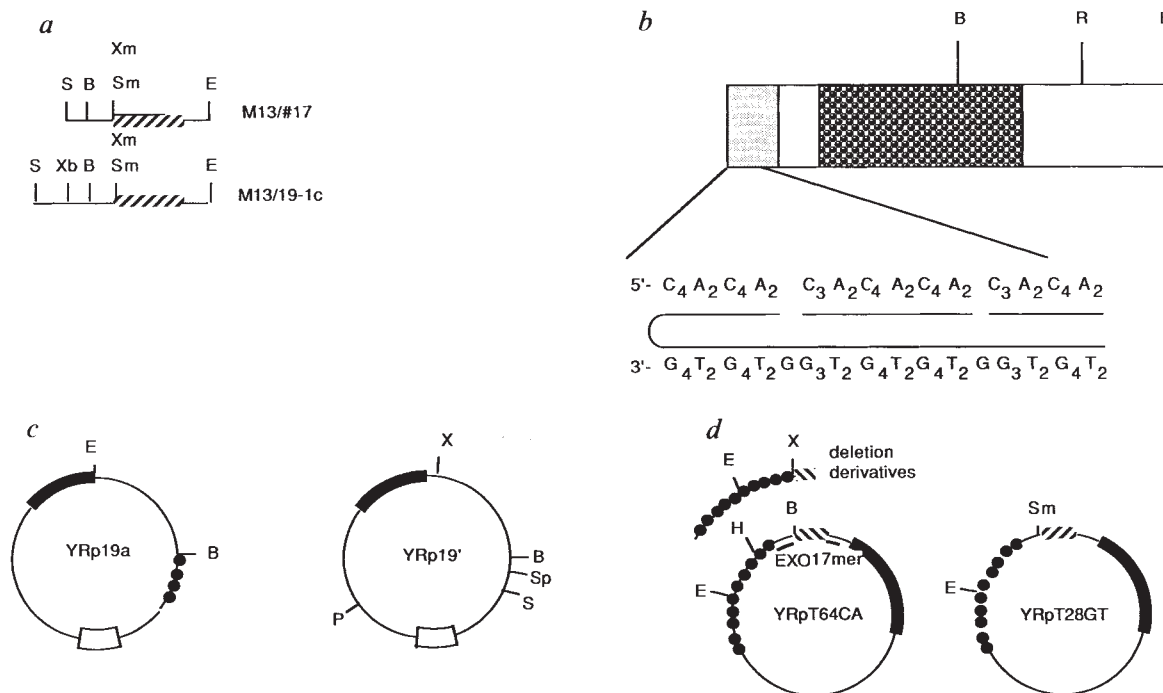
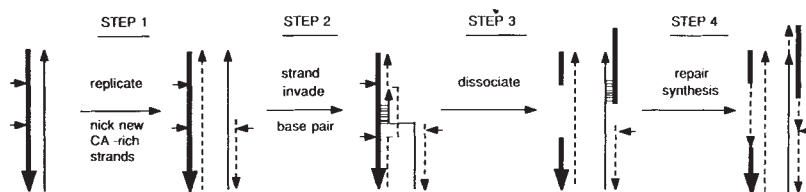


Fig. 2 DNA Structures. *a*, Cloned progenitors of test ends. The ~130-bp *SalI*-*EcoRI* restriction fragments from either M13/#17 (mp9) or M13/19-1c (mp19) were used to create test ends of various lengths and orientations. Both plasmids contain 112 bp of 5'(C₄A₄)_n3', indicated by the diagonally-striped box, where *n* was determined to be 14 by DNA sequence analysis. *b*, Control end. The terminal *Bam*HI (B) and *Eco*RI (R) restriction fragments of *Tetrahymena* rDNA were used as control end sequences on the chimaeric linear plasmids. The end of each consists of ~300 bp of C₄A₂. Adjacent unique sequences are indicated by the spotted box. Ribosomal DNA was isolated from *Tetrahymena thermophila* strain C3V (ref. 30). *c*, Vectors used to create YLp112CA, YLp112GTS and YLp112GTx. YRp19' is a 9.1-kb plasmid that contains *TRP1ARS1* (solid box) and *URA3* (open box). It was derived from YCp19 (ref. 31) by removal of the 1.5-kb *Xho*I fragment containing *CEN4*. YLp112CA was constructed by ligating test end sequences derived from M13/19-1c to the *Sal*I site and control-end sequences to the *Bam*HI site. YRp19a, which was used to construct YLp112GTS and YLp112GTx, is an 11.2-kb derivative of YRp19' in which the 187-bp *Bam*HI-*Sph*I portion was replaced with a 2.3-kb *Bam*HI-*Sph*I fragment of λ DNA (indicated by solid circles). *d*, Vectors used to construct YLps with test ends of 64 or fewer base pairs. The important feature of these vectors is that they contain test-end sequences in either of two orientations which are exposed as a free DNA terminus upon digestion with *Bam*HI (YRpT64CA), *Xho*I (deletion derivatives of YRpT64CA) or *Sma*I (YRpT28GT), and a single *Eco*RI site for ligation of control-end sequences. Symbols: solid box, *TRP1ARS1*; open box, *URA3*; solid circles, λ DNA; //, 5'(G₄T₄)_n3'; \\\, 5'(C₄A₄)_n3'; —, pBR322 or pUC. B, *Bam*HI; Bg, *Bgl*III; E, *Eco*RI; H, *Hpa*I; Hd, *Hind*III; P, *Pvu*II; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I; Xb, *Xba*I; Xm, *Xma*I.

Methods. *a*, M13/#17 was constructed by D. Gottschling by annealing two oligonucleotides, 5'-C₄A₄C₄A₄-3' and 5'-G₄T₄G₄T₄-3', such that out-of-register concatemers with four-base overhangs were produced. The unfractionated product was treated with T4 DNA polymerase to create blunt ends, and inserted into the *Sma*I site of M13mp9 such that the (+) strand of the phage DNA is the G₄T₄ strand. Plasmid 19-1c is the *Eco*RI-*Bam*HI fragment of M13/#17 inserted into *Eco*RI-*Bam*HI-digested M13mp19. Sequencing by the dideoxy chain termination method on alkaline-denatured²⁸ supercoiled plasmid templates was essentially as described²⁹ except that elongations were performed at 45 °C. *b-d*, For plasmid YRpT64CA, 130-bp *Sma*I-*Eco*RI fragment containing ~14 C₄A₄ repeats was isolated from M13/#17, treated with 5U *S*1 nuclease for 30 minutes at room temperature, and inserted into the *Sma*I site of pUC19. For plasmid YRpT28GT, the *Bam*HI-*Eco*RI fragment of M13/#17 was inserted into *Bam*HI-*Eco*RI-digested pUC19. Subsequent steps of the constructions were identical for the two plasmids. The *Eco*RI site in the polylinker was destroyed by treatment of *Eco*RI-digested DNA with T4 DNA polymerase and dNTPs to create blunt ends, followed by self-ligation. Termini of the 1.45-kb *Eco*RI fragment containing *TRP1ARS1* were rendered flush by treatment with T4 DNA polymerase, and inserted into the T4 polymerase-treated *Nar*I site of each plasmid. A 3-kb *Sph*I fragment of λ DNA, which contains a single *Eco*RI site, was inserted at the *Sph*I site in the polylinker of each plasmid. The resulting plasmids, YRpT64CA and YRpT28GT, are ~7.1 kb in length, contain unique *Bam*HI and *Eco*RI sites, and retain 64 bp of 5'(C₄A₄)₃' and 28 bp of 5'(G₄T₄)₃', respectively. Position of oligonucleotides (EXO and 17-mer) used for hybridization probes and sequencing primers are indicated. Deletion derivatives of YRpT64CA were constructed by limited digestion with Exonuclease III (ref. 32) from the single *Hpa*I site in the λ DNA. Blunt ends were created by treatment with T4DNA polymerase and dNTPs. The DNAs were then ligated to *Xho*I linkers, and following *Xho*I digestion, gel purification and self-ligation, were used to transform *E. coli*. The resulting transformants were subjected to three successive screens to identify plasmids with fewer than 64 bp of C₄A₄ as well as the necessary restriction sites for construction of chimaeric linear plasmids: (1) sequential colony hybridizations³³ with end-labelled EXO III probe and with the M13 17-mer sequencing primer, (2) DNA was prepared from colonies that hybridized to the 17-mer but not the EXO III oligonucleotide probe and analysed for the presence of both the *Xho*I and *Eco*RI sites, and (3) plasmids containing both an *Xho*I and *Eco*RI recognition site were sequenced to determine the number of C₄A₄ repeats.

observed.

Some unexpected results were obtained with both the control-end and the test-end probes. In DNA from four of the five transformants (Fig. 3c, lanes 3–6), the control-end probe also hybridized to a heterogeneous fragment with an average size of 3.2 kb, the same size fragment detected when the DNA samples were hybridized with the (G₄T₄)_{4.5} probe (Fig. 3b). These results indicate that the test end on four of the five plasmids contains C₄A₂ repeats in addition to the expected C₄A₄ repeats. The possibility that these results are due to ligation of the *Tetrahymena* terminus to both ends of the plasmid was eliminated by demonstrating that only the C₄A₂ repeats and none of the adjacent unique DNA in the *Tetrahymena* fragment hybridizes to the 3.2-kb fragment (Fig. 3d). In addition to the unexpected results with the control-end probe, in DNA from one of the transformants, the test-end probe unexpectedly displayed hybridization to a fragment of 8.3 kb (Fig. 3b, lane 6) indicating that the control end of this plasmid bears C₄A₄ as well as C₄A₂ repeats. Transfer of control-end sequences to the test end has been observed for the majority (90 per cent) of correct sized linear plasmids regardless of the length of the test end (Table 1). On 20 per cent of the plasmids examined, C₄A₄ repeats were detected on the end of the plasmid bearing the control terminus.

The terminal location of the telomeric sequences was established by monitoring their disappearance after digestion with the exonuclease BAL-31. BAL-31 digestion was carried out on total DNA from a strain carrying YLp112CA-5, a linear plasmid in which C₄A₂ sequences were detected on both the control and test ends of the plasmid (Fig. 3, lane 5). After BAL-31 digestion, the DNA was digested with *Xho*I which produces a 3.1-kb fragment containing the control end and a 7.0-kb fragment containing the test end (before C₁₋₃A addition). *Xho*I digestion of yeast chromosomal DNA produces a heterogeneous fragment of ~1.3 kb derived from Y'-bearing telomeres, as well as many other telomeric and non-telomeric fragments⁷. Figure 4 shows that the transferred C₄A₂ repeats, like the other telomeric sequences, are BAL-31 sensitive and therefore close to the plasmid end.

Total yeast DNA from a strain containing YLp112CA-5 (Fig. 3, lane 5) was digested with *Bgl*II (which removes the control end, Fig. 1c), self-ligated and used to transform *Escherichia coli*. A subclone carrying the test end of YLp112CA-5 was digested with *Bam*HI and then for increasing amounts of time with BAL-31 to determine the order of the telomeric sequences relative to the end of the plasmid (Fig. 5). Because the relevant *Bam*HI site in YLp112CA-5 is in vector DNA immediately adjacent to the test end, those sequences closest to the physical end of YLp112CA-5 should be the most resistant to the exonuclease. This analysis demonstrates that C₁₋₃A sequences are the most distal sequences on YLp112CA-5 followed by the transferred C₄A₂ repeats and then the C₄A₄ repeats, a result confirmed by DNA sequencing (S.-S. Wang, unpublished data). Therefore, transfer of control-end sequences probably occurs before C₁₋₃A addition.

What is the mechanism by which the terminal sequences are transferred between DNA termini? The transfer is not a ligation artefact because it involves only terminal CA-rich sequences, not the adjacent unique sequence DNA (Figs 3, 4). Transfer cannot be the result of the asymmetric resolution of a circular intermediate formed by end-to-end fusion of the starting plasmid in yeast to give a circular plasmid with chimaeric inverted repeats²⁰; asymmetric resolution of such a plasmid produces a DNA terminus carrying terminal sequences in the reverse orientation, which cannot function as a telomere in yeast (Table 1). Finally, if telomerase exists in yeast it would be expected to add C₁₋₃A repeats, but not non-yeast telomeric repeats¹⁹. Therefore, the most reasonable interpretation of these data is that during telomere formation, DNA termini usually undergo recombination.

Table 1 Formation of linear plasmids

Plasmid	Per cent correct	Number analysed	Number transferred
YLp112CA	56	48 (13)	12/15
YLp64CA	35	20 (11)	7/7
YLp48CA	22	23 (3)	5/5
YLp40CA	9	35 (12)	4/4
YLp28CA	6	64 (42)	3/3
YLp16CA	0	148*	NA
YLp10CA	0	275*	NA
YLp4CA	0	264*	NA
YLp0CA	0	94	NA
YLp112GTX	0	54	NA
YLp112GTS	0	81*	NA
YLp28GT	0	218†	NA
YLp112CA/ <i>rad52</i>	50	8† (100)	3/4

Each yeast linear plasmid (YLp) is defined by the number of base pairs and the orientation of the test-end sequences it carries (see text). The terminal structure of the test end is the DNA terminus generated by digestion with the following restriction enzymes: *Eco*RI (YLp112CA), *Bam*HI (YLp64CA), *Xma*I (YLp112GTX), *Sma*I (YLp112GTS, YLp28GT), *Xho*I (all other YLps). YLp112CA was constructed by simultaneous ligation of the 8.8-kb gel-purified vector (*Bam*HI-*Sal*I digested YRp19') with the gel-purified 130-bp *Eco*RI-*Sal*I fragment isolated from 19-1c containing (C₄A₄/G₄T₄)₁₄ and with the gel-purified 1.3-kb terminal *Bam*HI fragment from *Tetrahymena* rDNA (see Fig. 2). All other YLp(n)CA plasmids were constructed by double digestion of YRpT64CA (or deletion derivatives thereof) with *Eco*RI and with the enzyme indicated above to expose test end sequences. The resulting fragment (~5.8 kb) was gel-isolated and ligated to gel-isolated 2.6-kb terminal *Eco*RI fragment from *Tetrahymena* rDNA. In most experiments, ligation mixes were introduced into strain 34c (*MATa met2, his3V-1, leu2-3, leu2-112, tripl-289, ura3-52, cir⁰*) by lithium acetate transformation. For the *RAD52* experiments, DNA was introduced into strain XS95-6C (*MATa, rad52-1, ura3-52, trpl, his3V-1, leu2-112, 3; from D. Schild*) by spheroplast transformation. All of the *rad52* transformants containing linear plasmids retained hypersensitivity to X irradiation. Total undigested DNA isolated from transformants was analysed for the presence of plasmid sequences by Southern hybridization with ³²P-labelled pBR322. The percentage of transformants analysed which contained linear plasmids of the predicted size and structure is indicated by per cent correct. Other classes of transformants were those containing circular plasmids, no plasmids (that is gene conversion), or plasmids larger than 23.7 kb most of which did not hybridize to the test-end probe. When control termini are ligated to both ends of the vector, the per cent correct is similar to results obtained with YLp112CA. Unless otherwise noted, values reflect data collected from a single experiment. For constructs producing no linear plasmids of the correct size, nearly every transformant was analysed. For those constructs which produced linear plasmids of the correct size, the percentage of the total number of transformants which were analysed is indicated in parentheses (under number analysed). The number of linears of correct size that displayed transfer of terminal sequences over the number of linears of correct size examined is presented (number transferred). NA, not applicable.

* Data pooled from two independent experiments.

† Data pooled from three independent experiments.

The *RAD52* gene product is required for the majority of mitotic recombination events, including chromosome 'healing' by gene conversion²¹. The *RAD52* product is not required for sister chromatid exchange^{22,23}, nor for the addition of C₁₋₃A repeats on ciliate DNA termini^{21,24}. When the linear plasmid YLp112CA was introduced into a *rad52* strain, three of the four linears obtained exhibited transfer of C₄A₂ repeats to the test end (Table 1). Therefore, this telomere-telomere recombination must occur via recombination between the very ends of DNA molecules rather than by exchange of internal CA-sequences that would involve *RAD52*-dependent double-strand break repair.

Fig. 3 Transfer of terminal sequences on a chimaeric linear plasmid. *a*, Predicted structure of YLp112CA. The linear plasmid has a predicted size of ~10 kb and a single *PvuII* site which, when cut, produces a 2.9 kb fragment containing the test end and a 7.2 kb fragment containing the control end (before $C_{1-3}A$ addition, which is expected to add ~500 bp per terminus). *b-d* *PvuII* digestions of total DNA isolated from transformants containing linear and circular plasmids. *b*, Hybridization with $(G_4T_4)_{4.5}$, the test-end probe, and *c*, $(G_4T_2)_7$, the control-end probe. *d*, The probe was removed from the blot shown in *c* and the filter was rehybridized with the unique sequence probe for the control end. Lanes 1-6 contain, respectively, total DNA from yeast transformants carrying YRp19', YLp112CA-1, YLp112CA-3, YLp112CA-4, YLp112CA-5 and YLp112CA-6. Controls are lane 7, pTCS (obtained from A. Murray), digested with *XhoI* and *BamHI* to give a fragment (~300 bp) containing $(C_4A_2)_{50}$; lane 8, pof1 (cloned *Oxytricha* macronuclear rDNA (ref. 34)) digested with *PstI* to give three fragments, two of which (a 6.7-kb fragment and a 0.9-kb fragment) contain 30-35 bp (C_4A_4) . Size markers indicated by horizontal lines are *HindIII*-digested lambda DNA.

Methods. Total yeast DNA was isolated³⁵, fractionated on two identical but separate gels and transferred to nitrocellulose. Hybridizations using end-labelled oligonucleotide probes were performed at 42 °C in 50% formamide, 5×SSC, 1×Denhardt's³³, 1 mg ml⁻¹ heat-denatured herring sperm DNA and 10% dextran sulphate for 8-15 hours. Hybridization conditions using nick-translated probes are described³⁶. Nitrocellulose filters were washed at room temperature for 30 min in 5×SSC, followed by a 50 °C wash for 1 h in 5×SSC, 0.1% SDS. The 1,050-bp *HindIII* fragment isolated from pRP7 (ref. 37) which contains the non-coding region near the end of the *Tetrahymena* rDNA molecule was subcloned into M13mp19 and used as the unique sequence probe for the control end.

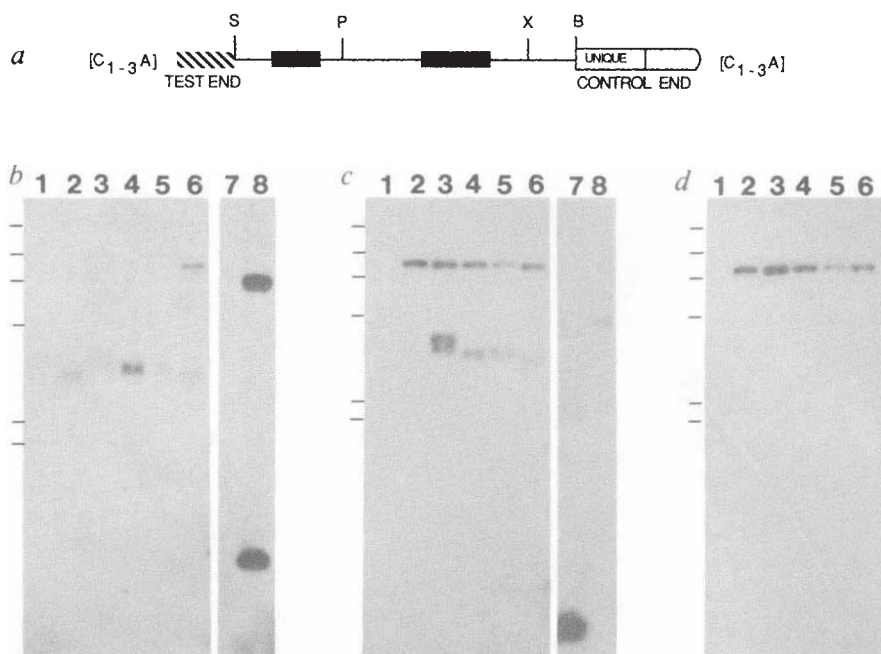
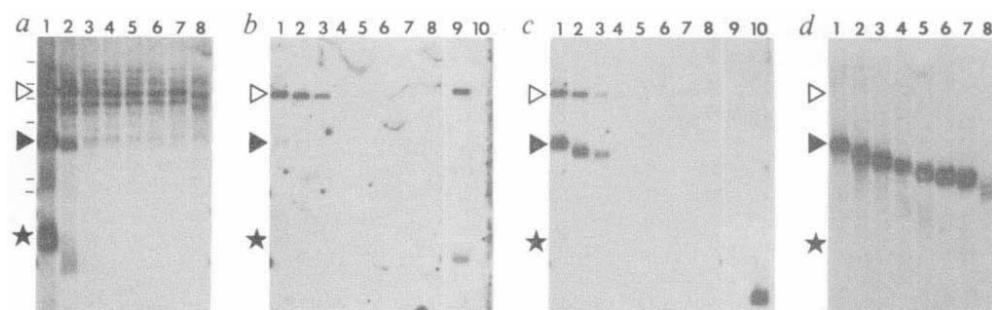


Fig. 4 Transferred terminal sequences are sensitive to BAL-31 digestion. Total DNA from a strain produced by transformation with the linear plasmid YLp112CA-5 (Fig. 3, lane 5), was treated with BAL-31. Probes are $C_{1-3}A$ (*a*); $(G_4T_4)_{4.5}$ (*b*); $(G_4T_2)_7$ (*c*) and control-end unique sequence (*d*). Lanes 1-8 contain, respectively, DNA removed after 0, 10, 20, 30, 40, 50, 60 and 120 minutes of BAL-31 digestion; lane 9, *PstI*-digested pof1 (contains C_4A_4 sequences); lane 10, *XhoI*-*BamHI* digested pTEC1.6 which yields a fragment of ~700 bp containing $(C_4A_2)_{50}$. Size markers indicated by horizontal lines are *HindIII*-digested lambda DNA. The open arrow points to fragments carrying the test end, the closed arrow to fragments carrying the control end, and the star to terminal fragments from Y' bearing chromosomal DNA molecules.



Methods. Samples were taken over a 2-h period, digested with *XhoI*, subjected to electrophoresis and sequential Southern hybridization. The $C_{1-3}A$ probe was prepared by gel-isolation of a 125 bp fragment which contains 81 bp of cloned $C_{1-3}A$ sequences derived from a yeast telomere⁸.

Discussion

Using linear plasmids in which each terminus is marked with a different telomeric sequence, we have shown that cloned telomeric sequences 28 bp or longer support telomere formation in yeast. Although termini as short as 28 bp can support telomere formation, the number of correct sized linear plasmids recovered is small (≤ 9 per cent) with test ends shorter than 48 bp (Table 1). During formation of new telomeres, telomeric sequences usually undergo recombination by a process that does not require the *RAD52* gene product. Thus, although C_4A_4 and C_4A_2 repeats share only limited homology, these sequences are presumably capable of base pairing *in vivo*, possibly facilitated by the ability of these sequences to adopt unusual DNA conformations²⁵ and/or by a RecA-like protein that promotes strand exchange²⁶. Precedent for base pairing of telomeric repeat sequences with nonhomologous but related DNA sequences is

provided by the ability of poly(dGT) to hybridize under high stringency conditions to yeast $C_{1-3}A$ sequences^{6,7}.

What is the *in vivo* role for this telomere-telomere recombination? Recombination could be a consequence of repair or maintenance processes although its *RAD52*-independence argues against this interpretation. Alternatively, the telomere-telomere recombination may be linked to telomere formation in which case it might also be the mechanism by which $C_{1-3}A$ repeats are added to ciliate termini. Telomere recombination can be visualized in terms of a model for telomere replication¹⁴ (Fig. 1): after replication the 3' overhang produced by primer excision at the end of the chromosome is able to strand invade and base pair with a complementary region on another strand. In our system, this recombination could occur between the two ends of the same plasmid or between termini on different DNA molecules of these multicopy plasmids. Subsequent strand dissociation is

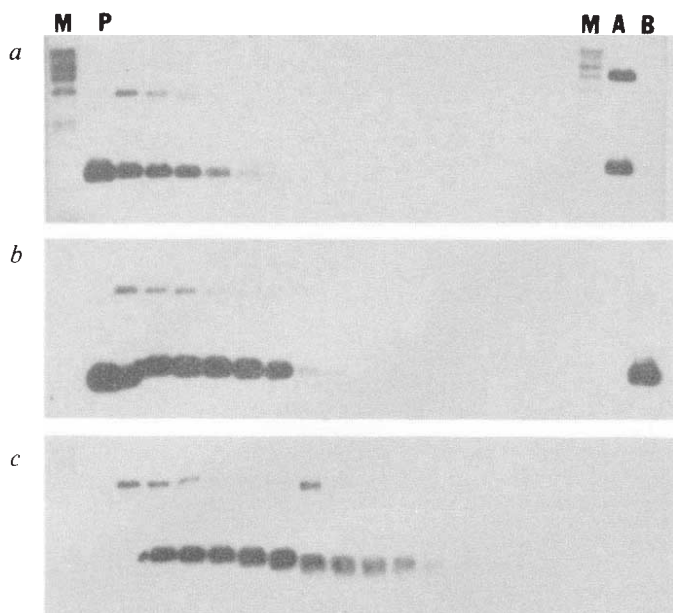


Fig. 5 BAL-31 digestion of cloned telomere. A recombinant DNA plasmid containing the test end of the yeast propagated plasmid YLp112CA-5 (Fig. 3, lane 5) was digested with BAL-31 to determine the order of the telomeric sequences. Southern blots were probed successively with: $(G_4T_4)_{4.5}$, *a*; $(G_4T_2)_7$, *b*; and $C_{1-3}A$, *c*. The lanes contain: M, *Hind*III-digested λ DNA; P, *Hind*III-*Bam*HI digested tc-0-6; A, *Pst*I-digested p0f1 DNA (C_4A_4), and B, *Xho*I-*Bam*HI digested pTEC1.6 DNA containing $(C_4A_2)_{50}$. The rest of the lanes from left to right contain samples digested for increasing amounts of time with BAL-31.

Methods. Total DNA from yeast containing YLp112CA-5 was digested with *Bgl*II, treated with T4 DNA polymerase in the presence of dNTPs and self-ligated. Digestion of YLp112CA with *Bgl*II removes the control end terminus (Fig. 2c), and self-ligation produces a plasmid (called tc-0-6) containing test end sequences. Plasmid tc-0-6 was first digested with *Bam*HI (the *Bam*HI site is derived from the poly-linker of M13/19-1C and is <10 bp from the C_4A_4 repeats) and then digested with BAL-31. Aliquots were removed at 5-min intervals during the first 0-30 minutes of digestion and then at 2-min intervals thereafter. Samples were digested with *Hind*III to release a fragment of ~1 kb containing the sequences from the test-end of YLp112CA, and subjected to electrophoresis. A portion of the nitrocellulose (containing *Hind*III-*Bam*HI digested tc-0-6 and the zero time point) was lost during washing from panel *c*. In each plasmid lane, the upper band is partially digested plasmid.

facilitated by specific nicks in the CA-strand. Any remaining gaps are closed by repair synthesis. Recombination by this mechanism is expected to be *RAD52*-independent because it does not involve repair of double-strand breaks. If recombination is necessary for telomere formation, the frequency of telomere formation is expected to be related to the length of the recombining end. Consistent with this, as the test end is decreased in size, the efficiency of telomere formation drops (Table 1). However, the fraction of plasmids of the correct size on which we detect transfer of terminal sequences is independent of the length of the test end (Table 1). Because nicks are concentrated in the CA-strand¹, the orientation requirement can also be explained by a recombination model. With telomere sequences in the reverse orientation, the invading strand is now the CA-strand instead of the GT-strand, and dissociation of the base-paired segment would require additional enzyme activities. The fact that nicks are concentrated in the CA-strand could also explain why we more often detect transfer of C_4A_2 repeats to the test end: the test end lacks single-strand nicks and therefore is expected to be an inefficient donor compared to the control end. Alternatively, the shorter test end may be an inefficient donor of terminal sequences because it (1) offers a smaller target for base pairing with the invading strand or (2) the amount transferred from this shorter tract is usually too little to be detected by hybridization.

We have demonstrated that during telomere formation in yeast, DNA termini often undergo recombination with other DNA termini. This system is the first in which there is a detectable consequence of recombination between the very ends of DNA molecules that can be distinguished from the consequences predicted by telomerase-mediated DNA replication. The question remains as to the *in vivo* role, if any, of telomere-telomere recombination. For example, the role of recombination among the terminal repeats may be to maintain their homogeneity²⁷. Alternatively, recombination might be involved in telomere replication, either in concert with telomerase or not. The ultimate test of these possibilities awaits the identification and characterization of mutants defective in maintenance of linear chromosomes. If recombination is important for telomere replication, some of these mutations are likely to be in genes encoding *recA*-like proteins, which like the T4 *uvrX* protein¹¹, promote synthesis between terminal sequences.

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Search for strange matter by Rutherford backscattering

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According to a number of suggestions, stable strange matter could exist in the form of supermassive nuclei (or 'strange nuggets')^{1,2}. In contrast to ordinary nuclei, which contain only 'up' and 'down' quarks, a piece of strange matter should comprise a mixture of 'up', 'down' and 'strange' quarks in roughly equal proportions. Small amounts of strange matter could have survived from the early stages of the Universe¹. Alternatively, strange matter might reach the Earth as a flux of strange nuggets produced in collisions of neutron stars³. Limits to the cosmic flux of strange nuggets with masses in the range from 10^{-4} to 250 g have been obtained in a search for light produced by the nuggets in the upper atmosphere⁴. Here we report the results of a search for supermassive nuclei by using Rutherford backscattering of heavy ions. The method is sensitive to a broad range of masses extending to those that exceed the projectile mass by several orders of magnitude. Upper limits for the abundance of strange nuggets with masses $A \approx 4 \times 10^2$ to 10^7 AMU relative to the number of nucleons were found to be in the range 10^{-10} to 10^{-14} .

To set a useful sensitivity limit for experiments designed to search for strange matter, De Rújula and Glashow estimated the maximum concentration of strange nuggets in the Earth's crust³. They assume a value of $\sim 10^{-24}$ g cm⁻³ for the local dark matter density of our Galaxy. Assuming that the whole of dark matter consists of strange nuggets, this flux corresponds to an annual Earth infall of strange matter of $\sim 10^9$ g. Over the Earth's 4.6-Gyr history this results in a maximum crustal concentration of 10^{-7} by mass. For the mass range considered in our experiment, $A \approx 4 \times 10^2$ to 10^7 , this limit extends from 3×10^{-10} down to 10^{-14} nuggets relative to the number of nucleons. Because nuggets with these masses are already stopped in the Earth's atmosphere³ they should not be distributed equally over all of the crust but should be concentrated in its upper layer, in which region the local abundance of nuggets may therefore be higher by two to three orders of magnitude.

In our experiments natural samples were irradiated with beams of ^{238}U and ^{208}Pb nuclei provided by the heavy-ion accelerator UNILAC in Darmstadt. The bombarding energy was 1.4 MeV per nucleon; this is far below the Coulomb barrier of nuclei, so that the cross-section for nuclear reactions is negligible⁵, and the interaction of nuclei is dominated by elastic scattering. In this case the angle and the velocity of the scattered projectile nucleus are determined by the mass of the target nucleus. In a single scattering event a projectile can be scattered into the backward hemisphere only by a heavier nucleus. Therefore, scattering of ^{238}U or ^{208}Pb through angles of 90° – 180° would indicate the presence of more massive and possibly strange nuclei.

Scattered nuclei were detected by a system of twelve low-pressure multi-wire proportional counters⁶ (Fig. 1) divided into four identical modules, each of which consisted of one start- and two stop-detectors. The ranges of scattering angles covered by this system are 92° – 120° and 127° – 170° , with a total solid angle of 1.15 sr.

The identification of detected nuclei is based on the following

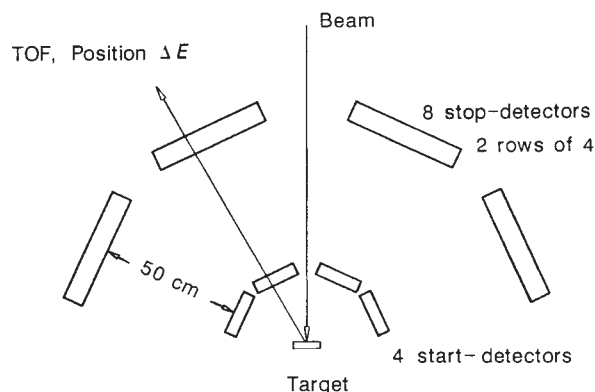


Fig. 1 Schematic diagram of the detection system. Four modules of multi-wire proportional counters are installed symmetrically around the beam axis (top view). Each detector module consists of a single start- and two stop-detectors, placed one above the other.

quantities. The time-of-flight (TOF), or equivalently the velocity, of each scattered nucleus is calculated from the time signals of start- and stop-detectors. The specific ionization (ΔE), which depends on the atomic number Z and the velocity v of the nuclei according to $\Delta E \propto (Z/v)^2 \ln(v^2)$, is measured by the stop-detectors, and, by calibrating ΔE against TOF, ΔE therefore characterizes the atomic number of each nucleus (Fig. 2). The trajectory and, in turn, the scattering angle is obtained from the position measurements in the start- and stop-detectors.

A target of ~ 1 – 2 mm thickness, prepared from an iron meteorite (Sam's Valley meteorite, Medford, Oregon, 1894), was irradiated with 1.7×10^{14} particles of ^{238}U . Figure 2a and b shows the two-dimensional spectra, in terms of ΔE as a function of TOF, of particles in the angular regions 92° – 120° and 127° – 170° , respectively. No data were recorded below $\Delta E \approx 110$ (in relative units) because of an electronic cutoff.

The solid curves in Fig. 2a, b represent the 2σ range (95.4% probability) of ΔE -TOF values for ^{238}U scattered from a thin uranium target in a calibration experiment. No scattered uranium nuclei were recorded for a TOF below 90 ns. Some uranium nuclei with a TOF greater than 90 ns are seen at 92° – 120° (Fig. 2a), but no uranium nuclei were recorded at angles exceeding 127° (Fig. 2b). This strong decrease in the number of scattered nuclei with angle is typical of multiple scattering, and for this reason the events with TOF > 90 ns were excluded from the analysis. The absence of events between TOF ≈ 30 ns, which corresponds to the initial velocity of uranium nuclei, and 90 ns can be used to estimate limits for the abundance of supermassive nuclei in a certain mass range. The lower limit of this mass range is determined by TOF = 90 ns, which corresponds to backscattering of uranium from nuclei with mass $A \approx 400$ located at the target surface.

A group of scattered nuclei with TOF centred around 70–80 ns, with specific ionization much lower than that for uranium ions of the same velocity, was observed at angles of 92° – 120° (Fig. 2a). From comparison with the calibration curves, this group was identified as comprising nuclei with $Z \approx 30$, which we thus attribute to iron nuclei. The dashed curve in Fig. 2a shows the loci of iron nuclei as calculated from tabulated energy-loss values⁷. As iron is the main component of the meteorite, an observation of scattered iron nuclei is not surprising. Such events can result from rescattering of iron recoil nuclei produced in primary uranium-iron collisions. We emphasize here that the identification of nuclei does not require any knowledge of the underlying scattering process (single or multiple), but is based entirely on the experimental calibration curves of ΔE against TOF.

To calculate upper limits for the abundance of supermassive nuclei with mass A , the Rutherford-scattering cross-section and

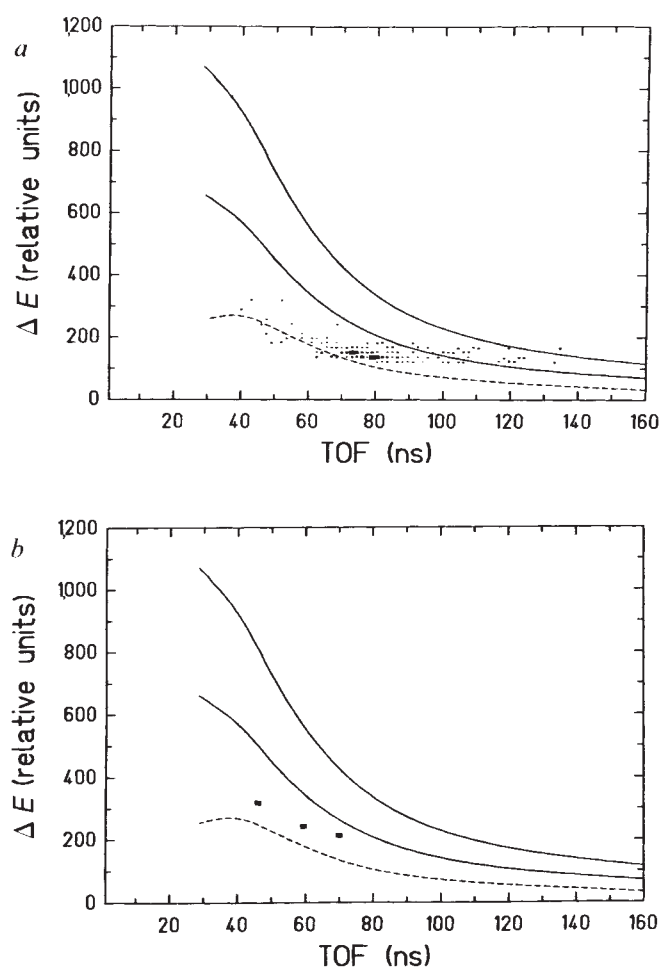


Fig. 2 Two-dimensional spectrum of specific ionization (ΔE) against time-of-flight (TOF) in the angular regions 92° – 120° (a) and 127° – 170° (b). The points represent the loci of a total of 245 (a) and 3 (b) detected particles. No data were recorded below $\Delta E \approx 110$. The initial projectile velocity corresponds to a TOF of 30 ns. The dashed line indicates the calculated locus of ^{56}Fe nuclei and solid lines span the 2σ range for scattered ^{238}U from a calibration experiment.

the effective target thickness must be known. The latter depends on the scattering angle and the mass A . The charge Z , as a function of A , of the supermassive nucleus determining the Rutherford cross-section was taken from the calculation in ref. 2 (with parameter values $E/A = 899$ MeV and $\alpha_c = 0.6$). With other values of these parameters, the upper limits decrease by up to a factor of 20, so that our estimate is model dependent. The Rutherford cross-section was calculated assuming point-like colliding nuclei, which is a good approximation for masses of (strange) nuclei up to 10^4 because the minimal distance between the nuclei is several times their size. For larger masses the minimal distance from the surface of the strange nucleus to the projectile becomes comparable with the size of the strange nucleus, which could necessitate corrections to the Rutherford formula. At present, our knowledge of strange nuclei is insufficient to enable such corrections to be calculated. The upper limits are shown by the solid line in Fig. 3 as a function of the mass number A of the supermassive nucleus. There is increasing sensitivity with increasing mass because of the corresponding increase in the Rutherford cross-section.

The screening of the nuclear charge by electrons inside a large strange nugget could be important, making an estimate of the effective charge difficult. As Farhi and Jaffe explain⁸, no appreciable screening occurs up to $A \approx 10^7$. We did not extend the

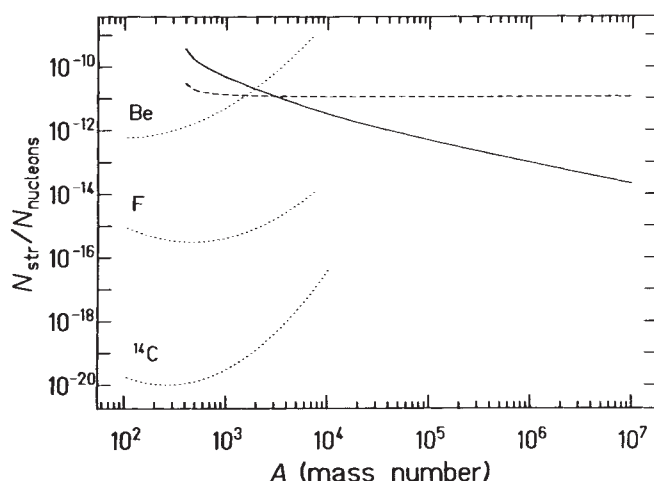


Fig. 3 Upper limits for the abundance of strange nuclei and supermassive relic particles ($N_{\text{str}}/N_{\text{nucleons}}$) as a function of the respective mass A . The solid line represents the upper limit for the abundance of strange nuclei from Rutherford backscattering of ^{238}U in an iron meteorite, and the dashed line shows the upper limit for the abundance of supermassive relic particles from Rutherford backscattering of ^{238}U in a lanthanide sample. Dotted lines show upper limits for the abundance of supermassive relic particles from a search for heavy isotopes of light chemical elements⁹.

estimated limits beyond $A \geq 10^7$ because above this, no reliable information on the screening was available. It is also important to note that the Coulomb barriers of strange nuggets are high enough⁸ (except for nuggets of low atomic number) that elastic scattering is dominant at energies of 1.4 MeV per nucleon.

In addition to the iron meteorite, several terrestrial samples were studied using ^{238}U and ^{208}Pb beams: garnets, manganese nodules and the group of lanthanide elements extracted from the minerals apatite, monazite and bastnesite. The combined upper limits, not shown, are two times lower than those for the iron meteorite.

The dotted lines in Fig. 3 show results of a search for supermassive isotopes of light chemical elements, using an electrostatic spectrometer⁹. These isotopes were expected to be ordinary nuclei containing supermassive particles that are relics of the Big Bang^{10–12}. The upper limits obtained for the abundance of supermassive isotopes with masses between 10^2 and 10^4 were rather low; we can consider these limits to be those for strange matter as well.

Our results, on the other hand, can be interpreted as giving upper limits for the abundance of supermassive relic particles that are bound to nuclei by hadronic interactions so that they cannot be detached in a collision process. Such limits are shown by the dashed line in Fig. 3 for relic particles attached to lanthanide elements. These limits can be considered as complementary to the previously published results (ref. 9 and references therein) because they cover a range of masses ($> 10^4$ AMU) which was not investigated in ref. 9.

From our present knowledge of strange matter² we do not expect its properties and abundance to fluctuate from one atomic number to the next. Thus, a further improvement in sensitivity can be achieved with chemically enriched targets, particularly by using chemical separations¹² of elements which do not occur naturally, such as technetium¹³, plutonium¹⁴ or promethium. Strange matter with atomic numbers of these elements should be as stable and as abundant as that of neighbouring atomic numbers. Thus, by chemically separating these elements from bulk material it is possible to obtain targets enriched in strange matter by several orders of magnitude. A similar reasoning applies to supermassive relic particles¹².

Our limits for the abundance of strange matter are close to the instructive estimates of De Rújula and Glashow³. At present we do not consider it useful to put constraints on these theoretical considerations, because, for the samples investigated here, we cannot exclude the possibility of appreciable fluctuations in the concentration of supermassive nuclei caused by fractionations during chemical processes in the early Universe. Further studies of primitive meteorites, which have not been subjected to gravitational effects and whose composition reflects the primordial abundance of nuclei, may provide appropriate data from which to extract such constraints.

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Growing drops of strange matter

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Strange matter comprises roughly equal numbers of up (u), down (d) and strange (s) quarks, in contrast to nuclear matter, for which s quarks are absent. The theoretical possibility¹⁻⁷ that S-drops (small 'bags' of strange matter) may not only be metastable, but might become absolutely stable if grown to sufficient size, would have consequences of the greatest importance. In particular, this form of matter could provide the basis for a compact energy source. Here we describe a hypothetical experiment to explore this possibility. The first step is the production and detection of S-drops in relativistic heavy-ion collisions. Previously, we have estimated⁸ the efficiency of production of S-drops by the process of fragmentation of quarks into hadrons following formation of hot quark-gluon matter. The proposed experiment would initially produce and detect S-drops of charge $Z = -1$ in present CERN and BNL facilities. The next step would be to grow small, positively charged S-drops to large stable S-drops through neutron capture in a confining apparatus. These two steps could provide the scientific basis for subsequent engineering studies of S-drops as an energy source.

Considerable theoretical interest has centred on the intriguing possibility that not only may S-drops be very long-lived, but large extended such objects, comprising strange matter (S-matter), might be absolutely stable. A nucleus of baryon number A consists of neutrons and protons, each of which is made up

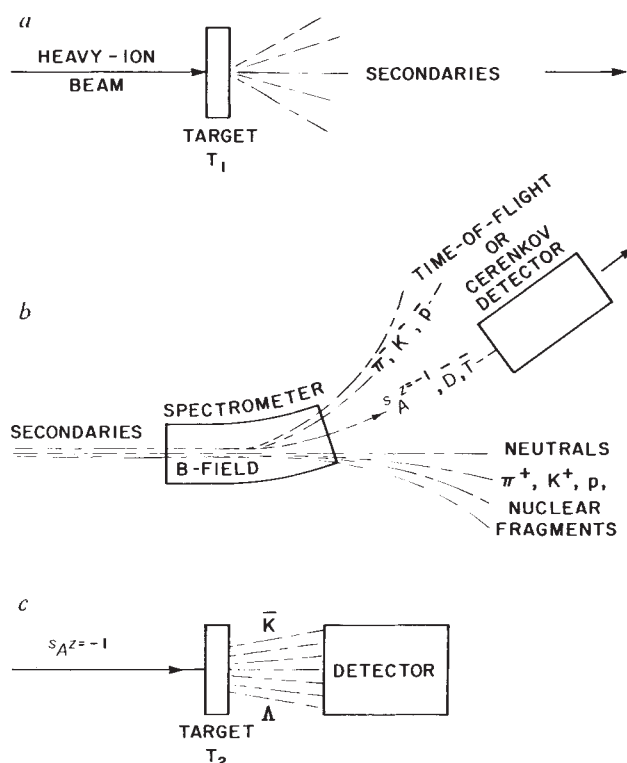


Fig. 1 A schematic diagram of the proposed high-sensitivity experiment to detect $Z = -1$ S-drops at CERN and BNL. *a*, Literally hundreds of secondaries will be produced in several types of processes on collision of the beam with target T_1 . *b*, S-drops $^{sA}Z = -1$ with momentum given by equation (1) are separated in the magnetic spectrometer. *c*, Positive identification of separated relativistic S-drops is made by secondary collisions in the downstream target T_2 , producing a large number of $\bar{K}$ and Λ particles.

of three (massless) non-strange quarks (udd and uud respectively) confined to a small 'bag'. Consider a single bag enclosing a total of $3A$ u and d quarks. This has a higher energy than the standard nucleus A . By replacing some u and d quarks by s quarks, however, the Fermi energies in the system can be lowered significantly. Neglecting the strange quark mass, m_s , the number of strange quarks n_s in this S-drop should be

$$n_s = n_u = n_d = A$$

Chin and Kerman³ calculated that S-drops with $A > 10$ and $n_s/A > 0.8$ would be metastable with lifetimes $\tau > 10^{-4}$ s. More recently, Witten⁵ made the startling suggestion that very large S-drops, indeed S-matter, with $n_s/A \approx 1$ might be absolutely stable, that is, the energy per baryon is < 938 MeV. This has important implications for S-drops: neutron stars might actually consist of S-matter with essentially no crust, a possibility with interesting astrophysical consequences^{5,9-13}. Of more general significance, however, is the possibility that a drop of stable S-matter could be used as an energy source, because, unlike nuclei, S-drops become more stable as they grow. It is this exciting idea that we wish to discuss here.

Using the Fermi-gas model with lowest-order quantum chromodynamics (QCD) corrections, Farhi and Jaffe⁶ calculated that S-matter could be stable for an appreciable range of values of the QCD coupling strength and of m_s . Bethe *et al.*¹⁴ argued against S-matter having a lower energy than nuclear matter. However, those authors approximated S-matter as being equivalent to an interacting gas of Λ particles, which is not a convincing model. As it seems likely that QCD calculations will not be accurate enough in the foreseeable future to give a definitive prediction on the stability of S-matter, experimental

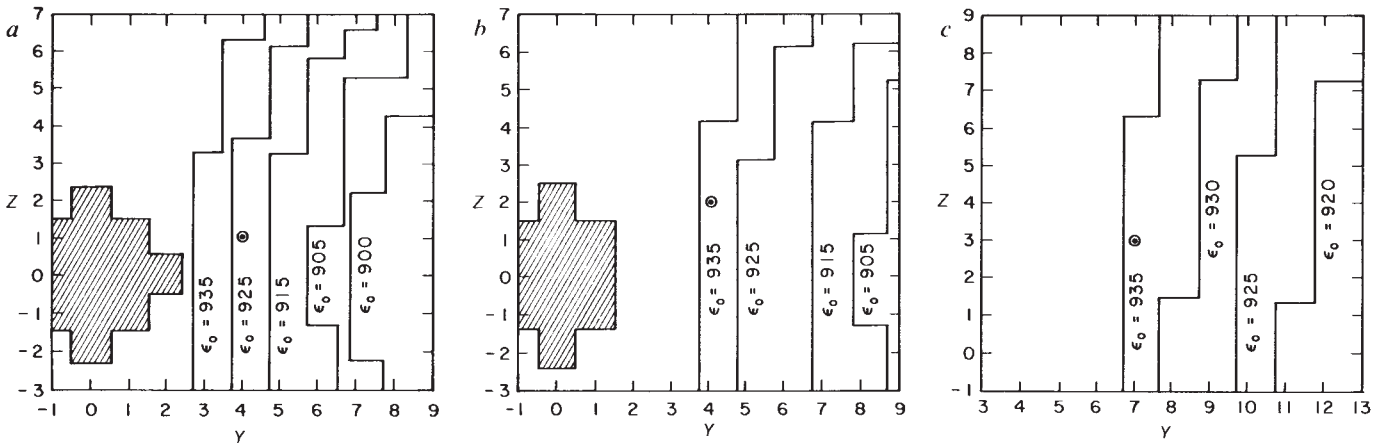


Fig. 2 Stability of S-drops against neutron emission by the strong and weak interactions for $m_s = 150 \text{ MeV}/c^2$, calculated using the mass relations of Berger and Jaffe⁷ for ${}^S A^Z$ as a function of ϵ_0 , the energy per baryon for stable infinite S-matter. *a*, For $A = 15$, the S-drop is stable against neutron emission ($\Delta Y = -1$, $\Delta Z = 0$, $\Delta A = -1$) by the strong interaction if (Y, Z) is in the region to the left of the solid line for a given ϵ_0 . Here, $Y = A + S$ is the hypercharge. For $\epsilon_0 \leq 920 \text{ MeV}$, the S-drop is also stable against neutron emission ($\Delta Y = 0$, $\Delta Z = 0$, $\Delta A = -1$) through the weak interaction for all (Y, Z) . The unstable region against weak neutron emission, for $\epsilon_0 = 925 \text{ MeV}$, is shown by the shaded region. The solid circle at $(Y, Z) = (4, 1)$ is the location of the lowest-energy $A = 15$ S-drop. This is an S-drop ${}^S A^Z = {}^{11}15^1$ with $n_d = 18$, $n_u = 16$ and $n_s = 11$. *b*, Same as *a*, but for $A = 20$. Again the shaded region corresponds to $\epsilon_0 = 925 \text{ MeV}$, and is a smaller domain than that in *a*. $(Y, Z) = (4, 2)$ is the energy minimum. *c*, Same as *a*, but for $A = 40$. For $\epsilon_0 = 925 \text{ MeV}$, there is no region unstable against weak neutron emission.

tests are necessary and warrant prompt attention in view of the significance of a positive outcome.

Consider step 1 of our proposed scenario, the production and detection of S-drops in the present relativistic heavy-ion-beam (fixed-target) facilities at CERN (200 GeV per baryon) and BNL (14.5 GeV per baryon). We assume that, as in the 'standard' picture of such collisions, a high-density, hot quark-gluon (QG) drop is formed¹⁵. We stress that the probability for forming such a QG drop need not be large and that a phase transition to a QG plasma need not occur for our proposal to be viable. (No real evidence for the formation of QG plasma has been found in the recent experiments¹⁶ at BNL and CERN.) Liu and Shaw⁸ calculated the emission of mesons and baryons in several (strong) interaction processes from a QG-drop precursor of an S-drop. The dominant process was the emission of high-momentum u and d quarks which 'fragment' and pick up an $\bar{s}$ quark from an $s\bar{s}$ pair to form a meson, leaving behind an s quark. (See also the mechanism for S-drop production of Greiner *et al.*¹⁷.) The formation of many strange particles is typical of the observed events at CERN. Although model-dependent, the possibility of producing an S-drop with $n_s/A > 0.8$ from the QG drop was much greater for small- A (≤ 30) drops, this probability being quite appreciable. We imagine that an S-drop cools to a metastable state ${}^S A^Z$ (each s quark contributing a strangeness $S = -1$) mainly by the emission of mesons and photons. A range of states ${}^S A^Z$ will be produced. Although the detection of these S-drops is conceptually simple (because of the low Z/A ratio), it is technically very difficult because of the huge multiplicity of particles produced in a head-on collision.

We propose that a search be made for $Z = -1$ S-drops at CERN and BNL. Consider an S-drop ${}^S A^{Z=-1}$ produced in the QG matter formed from the relativistic collision between a beam heavy-ion with energy $\gamma \text{ GeV}$ per baryon ($\gamma \gg 1$) and a fixed-target heavy nucleus (Fig. 1*a*). We assume that on average each nucleon in a beam-ion interacts with one nucleon in the target nucleus; this defines a rest frame for S-drop production. The laboratory momentum of the S-drop then is

$$P_S \approx c(\gamma/2)^{1/2}({}^S A^{Z=-1}) \text{ GeV}/c \quad (1)$$

in a nearly forward direction. The key to the separation of these S-drops from other products in the magnetic spectrometer in Fig. 1*b* is that if A is greater than some reasonable minimum

(~ 15) the S-drops will have a larger momentum than all the relevant background secondaries, specifically negatively charged π^- , K^- and $\bar{p}$. These latter particles, with mass m , would have momentum $P \leq c\gamma m$. In a field B , the spectrometer will bend particles in an arc of radius R according to $(P/Z) (\text{GeV}/c) = 0.3 BR$ (with B in tesla and R in metres), so that, taking $m = 1 \text{ GeV}/c^2$, for ${}^S A_{\min}^{Z=-1} > \sqrt{2}\gamma$, all negatively charged π^- , K^- and $\bar{p}$ will be bent more than the S-drops, which may thus be separated from these negatively charged particles (as well as from the neutral and positively charged secondaries). At CERN, heavy-ion beams¹⁶ having $\gamma = 60$ and 200 have been used, so that ${}^S A_{\min}^{Z=-1}$ will be 11 and 20 respectively. The only 'standard' background would come from $Z = -1$ antideuterons ($\bar{D}$) and antitritons ($\bar{T}$) which are produced in proton-nucleus collisions with rates¹⁸ $\bar{D}/\pi^- \leq 10^{-5}$ and $\bar{T}/\pi^- \leq 10^{-9}$. The BNL heavy-ion beam¹⁶ has $\gamma = 14.5$, and thus will not produce a $\bar{D}$ or $\bar{T}$ background. Central collisions might also produce negative, fractionally charged free quarks as a background; limits of $< 1.0 \times 10^{-9}$ quarks per incident oxygen ion have been set at BNL¹⁹. Following magnetic separation, Čerenkov detectors or time-of-flight detectors can then be used to distinguish between the ${}^S A^{Z=-1}$ drops and the low background of $\bar{D}$ or $\bar{T}$ at CERN. Finally, positive identification of these separated relativistic S-drops could be made in secondary collisions in the downstream target (Fig. 1*c*). The centre-of-mass energies will produce excitations much greater than the total binding energy of the S-drop, which will then disintegrate into a large number of $\bar{K}$ and Λ particles, giving a readily detectable and distinctive signature.

For an S-drop of given A , it is expected that the minimum-energy state will have positive Z . As can be seen from the mass relations of Berger and Jaffe⁷, S-drops with negative Z will not be stable. (Perhaps this is fortunate, as stable negatively charged S-drops might interact with normal matter to grow without bounds.) However, we see that, for a considerable range in parameters, the $Z = -1$ S-drops will live sufficiently long (Figs 2 and 3) to perform the high-sensitivity experiment depicted in Fig. 1. More quantitative details will be published elsewhere.

Assuming that the production of these small $Z = -1$ S-drops is indeed detected as outlined above, let us turn to step 2, the growing of S-drops for their use as an energy source. It is clear that an intermediate step would be to optimize the production

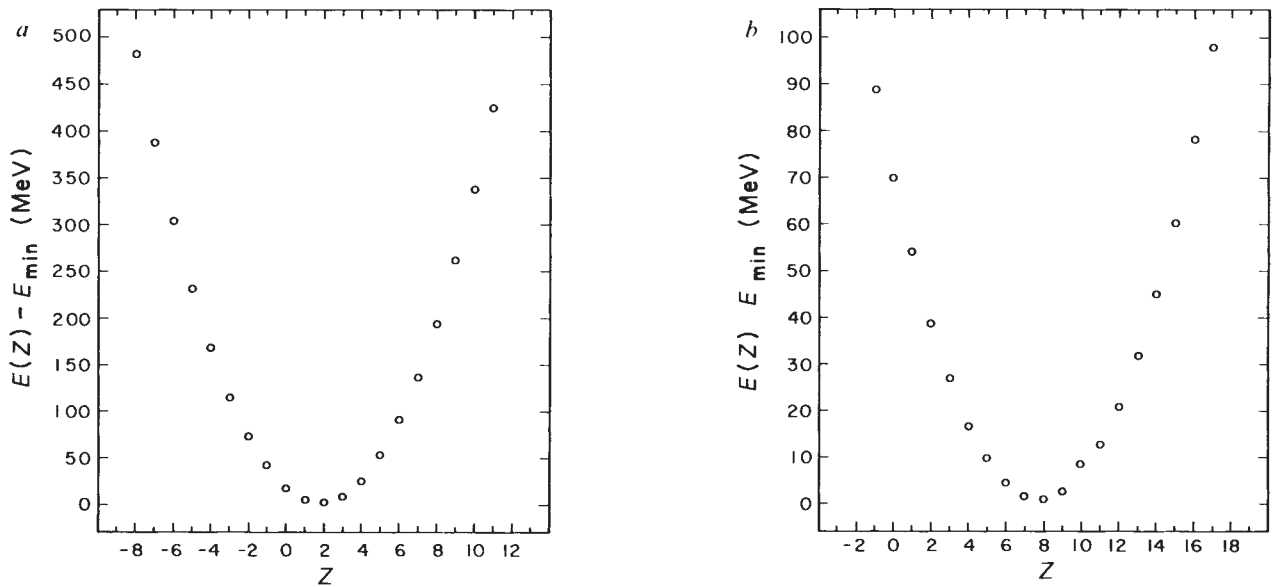


Fig. 3 *a*, Energy of the S-drop $E(Z)$, calculated using the mass relation of ref. 7, as a function of Z relative to its minimum value $E_{\min}$ ($\equiv E(Y=Y_{\min}=4, Z=Z_{\min}=2)$), for $A=20$, $Y=Y_{\min}$, $\epsilon_0=920$ MeV and $m_s=150$ MeV/ c^2 . This relative energy is rather insensitive to the choice of ϵ_0 (in the range 900–935 MeV). We note that an S-drop ${}^S A^Z = {}^{-16}20^{-1}$ would have an energy $\Delta E \approx 40$ MeV above the ground state ${}^{-16}20^0$, and $\Delta E \approx 20$ MeV above the ${}^{-16}20^0$ state to which it will decay by β -emission ${}^{-16}20^0 + e^- - \bar{\nu}_e$. By scaling tabulated β -decay rates, we obtain the lifetime $\Gamma_{\Delta Z=1, \Delta S=0} \approx 10^3 (\Delta E (\text{MeV})/20)^5 \text{ s}^{-1}$. Thus, this $Z=-1$ S-drop would live much longer than is necessary to perform the experiment proposed in Fig. 1. *b*, Same as *a*, but for $A=100$ (for which $Y_{\min}=19$ and $Z_{\min}=8$).

and separation of the metastable (positive- Z) S-drops. We note that experimental and theoretical examination of the S-drops so produced, and their decays, will provide important information concerning the nature of the mass formula. The energy per baryon of stable infinite S-matter, ϵ_0 , will thus be determined, as well as the quantitative details of the shell effects considered by Takahashi and Boyd²⁰.

The S drops produced and separated in the heavy-ion collisions should be grown as quickly as possible for at least the following reasons: (1) although stable against neutron emission for certain values of ϵ_0 (see Fig. 2), these S-drops might be unstable against α -decay, with lifetimes which, because of the Coulomb barrier, are long on experimental timescales (seconds) but not in relation to the needs for storage; (2) their binding energy per baryon grows with A ; (3) their neutron-absorption cross-section increases as $A^{2/3}$; (4) their 'sticking' probability to a surface increases with A (that is, their thermal velocity decreases). Thus we propose a technique for rapid growth of relatively small (perhaps $A \approx 30$) S-drops to considerably larger (perhaps $A > 10^4$) ones, by passing through liquid deuterium in a confining apparatus as shown in Fig. 4. The small relativistic metastable ${}^S A^Z = -1$ drops are slowed down in a linear electrostatic decelerator to non-relativistic velocities. Near the end of this decelerator would be a tank of liquid hydrogen of length such that an S-drop passing through it would pick up two protons and emerge with a charge $Z=1$. (Once $Z=-1$ S-drops are detected, one could infer that $Z>0$ S-drops are also being produced at still higher rates. A major effort should then be made to optimize the production and the separation of these $Z>0$ S-drops. This in turn would enable $Z>0$ S-drops to be injected directly into the decelerator, obviating the need for a liquid-hydrogen tank.) An emerging ${}^S A^Z = 1$ drop is then circulated through the liquid-deuterium tanks, where it picks up neutrons with a cross-section that we estimate (from nuclear (D, p) reactions) to be $\sim (A/30)^{2/3} 10^{-25} \text{ cm}^2$. An absorbed neutron in the S-drop would project onto its quark degrees of freedom and decay very rapidly by γ -transitions to the lowest allowed u and d levels. As neutrons are absorbed, the Fermi level of the d quarks increases relative to that of the s quarks, until a $d \rightarrow s$ transition takes place with subsequent γ -emission:

following neutron capture, an S-drop ${}^S A^Z$ will relax towards its energy minimum by strangeness-changing weak decay ${}^S A^Z \rightarrow ({}^{S-1} A^Z)^*$, which essentially changes a d quark to an s quark ($u+d \rightarrow s+u$) in a charged weak-current interaction. The asterisk indicates an excited state, which decays rapidly by γ -emission. Scaling the similar decay ($K^+ \rightarrow \pi^+ \pi^0$), we obtain a decay rate

$$\Gamma_{\Delta S=-1, \Delta Z=0} \approx 10^5 \left(\frac{\Delta E (\text{MeV})}{20} \right)^2 \text{ s}^{-1} \quad (2)$$

The $E(Y)$ curves required to evaluate (2) (where Y is the hypercharge $A+S$) are numerically very similar to the $E(Z)$ curves in Fig. 3.

Thus the S-drop grows and becomes more stable; we envisage small initial S-drops ($A \approx 30$ –50, for which $Z_{\min} \approx 3$) growing to a size $A \geq 10^4$. Our rough calculations of absorption length, Coulomb barrier, time constants and so on seem reasonable for

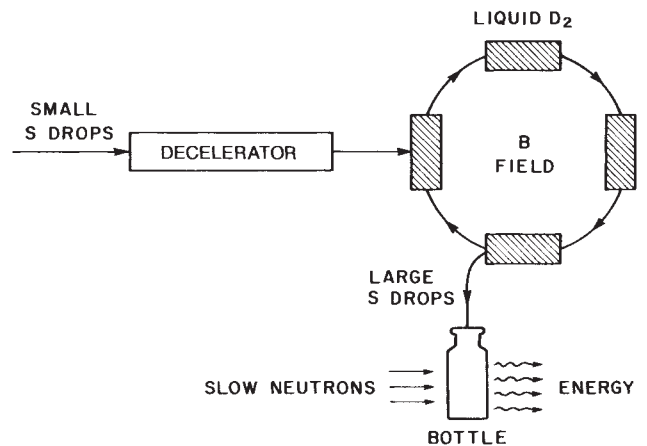


Fig. 4 Schematic diagram of proposed apparatus for the rapid growing of small S-drops into large S-drops. These can be stored and used as a compact energy source, by feeding in slow neutrons. For each neutron absorbed by an S-drop, $\sim (938 - \epsilon_0)$ MeV of energy is released (mostly in γ -rays).

the values $B \approx 1$ tesla, S-drop velocity $v/c \approx 0.1$ and a radius for the ring of tanks of ~ 5 m. Some acceleration of the S-drop between liquid-deuterium tanks would be necessary to maintain $v/c \approx 0.1$. The large- A S-drop may then be put into a ‘conventional’ storage bottle, where it decays to its stable $Z_{\min}$ and $Y_{\min}$ for that A . (Note that if we start from small Z values, and keep always below $Z_{\min}$, the Coulomb barrier for the S-drop will inhibit the possibility of α -decay until it has grown sufficiently large to be stable against such decay.) These large stable S-drops could then be used as a compact energy source.

Because stable S-matter may exist for a range of QCD parameters, one might hope to be able to investigate its formation experimentally. Our proposal, outlined schematically in Fig. 1, for producing and detecting small ${}^A Z=-1$ drops could be carried out readily in present CERN and BNL relativistic heavy-ion fixed-target facilities. Our next proposal, outlined in Fig. 4, for rapid growth of these small S-drops into larger ones, to be stored and used subsequently as an energy source, seems plausible. We emphasize that our calculations of energies and lifetimes, given in Figs 2 and 3 and based on mass relations⁷ for ${}^A Z$, should be considered as only illustrative. No fine tuning of parameters is necessary for our general scheme to be viable. The use of the stable S-drops stored in the bottle (Fig. 4) as an energy source appears conceptually simple. Slow neutrons are fed into the bottle; for each neutron absorbed by a large S-drop, an energy of $\sim(938 - \epsilon_0)$ MeV is released, almost entirely in the form of γ -rays, and the S-drop becomes larger and more stable. In conclusion, therefore, we suggest that if S-matter is stable the production and growth of stable S-drops should be feasible and of considerable theoretical and pragmatic interest.

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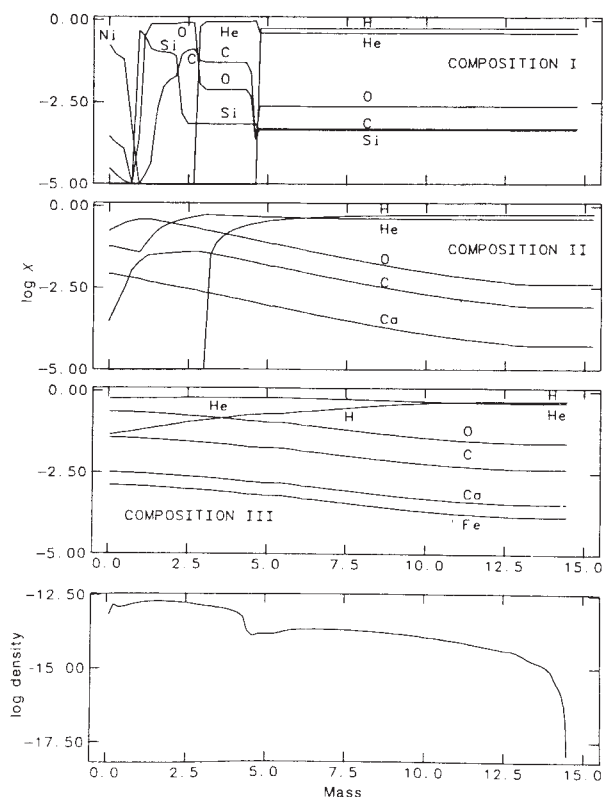


Fig. 1 Mass fraction X for several representative elements plotted logarithmically against mass for three mixed-composition models. Composition I (top) results from a standard one-dimensional hydrodynamic calculation. Compositions II (upper middle) and III (lower middle) are *ad hoc* mixed versions of composition I. The models correspond to models 10H (ref. 5), 10HM (ref. 9) and 10HMM (ref. 11), provided by S. Woosley (private communication). In the bottom panel is plotted the logarithm of the mass density against mass. Note the steep gradient near $5 M_{\odot}$, corresponding to the helium/hydrogen interface of the initial model, which indicates a natural delineation between ‘core’ and ‘envelope’. The neutron-star contribution has been removed from these plots and the mass coordinate rescaled appropriately.

model atmospheres. The new features of this calculation are the inclusion of a complex model hydrogen atom and the self-consistent treatment of an opaque lower boundary which approximates the observed continuum. We adopt hydrodynamic models reported in the literature, which are known to reproduce the observed light curve and γ -ray and X-ray spectra, and we obtain qualitative agreement with observed optical spectra. By examining the effect of mixing core material into the envelope, we conclude that the metals in the hydrogen-rich envelope make a substantial contribution to the optical nebular lines even for models with no outward mixing of the core material; however, a significant fraction of the energy emitted in ^{56}Co decay must be deposited in the envelope to produce the observed linewidths and hydrogen line strengths.

Synthetic spectral diagnostics have been applied to the late-time conditions in supernova 1987A by Fransson and Chevalier³. They assumed a stratified core composition, with the radioactive heating source confined to the central regions, and neglected the hydrogen envelope. Their calculations did not incorporate the possibility of mixing of the composition. Recent theoretical investigations conclude that a large amount of mixing of the composition, and particularly of the radioactive ^{56}Co heating source, is required to accurately reproduce the light curve⁴⁻¹⁰ and the γ -ray and X-ray spectral development¹¹⁻¹⁶.

Here we analyse the mixing scenario by presenting optical spectra from models with different degrees of mixing. We distinguish between 'composition mixing', which implies a smoothing of some part of the stratification that arises naturally in one-

Late-time optical spectra of SN1987A

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Optical study of late-time nebular emission provided the first direct evidence for the presence of radioactive ^{56}Co in the ejecta of Type Ia supernovae¹ and yielded conclusive evidence that Type Ib events constitute a physically distinct class of supernovae². Hydrogen-rich Type II supernovae have not been studied in detail in the nebular phase, but SN1987A provides an unprecedented opportunity to probe the structure and composition of the ejecta by modelling the nebular spectrum. Here we investigate the late-time nebular spectra of SN1987A by computing the emergent spectra from

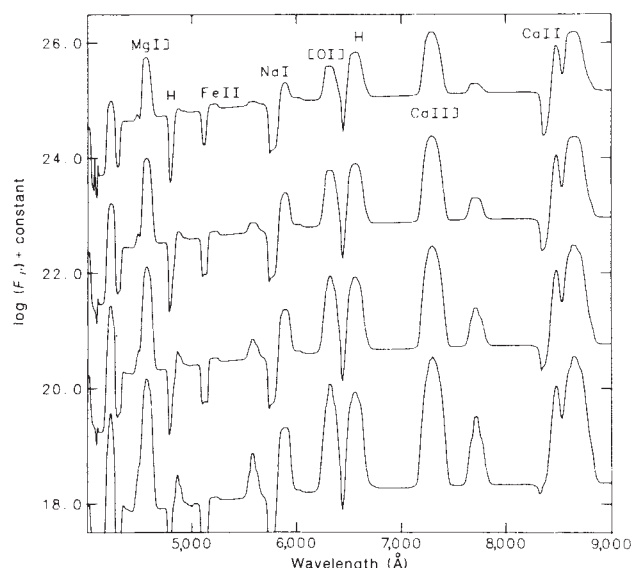


Fig. 2 Spectra for composition III at 200 days for various photospheric radii. From bottom to top, the radii are $R_{ph} = 1.5, 2.0, 3.0$ and 4.5×10^{15} cm, corresponding to mass points 1.1, 3.0, 4.6 and $7.5 M_{\odot}$. For clarity the spectra have been shifted relative to each other by two logarithmic units on the vertical axis. Note the contribution to the lines from the 'envelope' mass (upper spectrum). In the lower two spectra, spikes are evident at the centre of the line profiles, arising from contributions from the exposed, slow-moving core material.

dimensional hydrodynamic models, and ^{56}Co mixing', which can be imposed on the model while maintaining stratification of the remaining elements. The distinction between composition mixing and ^{56}Co mixing is necessary to clarify our conclusions. One might imagine that the mixing of ^{56}Co , without mixing of the other elements, appears contrived. The possibility exists, however, that freshly synthesized material created in the deepest layers could penetrate into the envelope in the form of small, energetic 'clumps', which could provide extensive ^{56}Co mixing while preserving the original stratification of the remaining composition.

The techniques used here for computing the late-time spectra are similar to those employed previously for supernovae^{1,17,18} and for SN1987A in particular³. The equilibrium structures of temperature and of ionization are found by imposing radiative and thermal equilibrium. Radiative equilibrium requires that heating of the gas must be balanced by radiation escaping the gas. It follows immediately that any given model will underestimate the total number of emission lines present in the real atmosphere and thus that the model can provide only an upper limit to the emissivity of the lines that are included.

We solve the radiative-transfer equations for the lines using the Sobolev approximation^{19,20} with modifications^{21,22} applicable to many-level and many-ion conditions. Special modifications necessary for solving the combined line-transfer and statistical-equilibrium equations for hydrogen will be discussed elsewhere (D.A.S., manuscript in preparation). We ignore continuum transfer. We do, however, mimic the effect of an optically thick inner region by imposing a lower boundary condition with a prescribed intensity I_{ν}^{inc} incident from below. The radius, R_{ph} , of this 'photosphere' and the frequency variation of the continuum are free parameters in the models. We require that the total luminosity L from the opaque region equals the true luminosity of the material that is heated radioactively below R_{ph} . We choose the continuum shape to be represented by a diluted black body at temperature T_{BB} , and define $L_{BB} = 4\pi R_{ph}^2 \sigma T_{BB}^4$. The dilution factor $\delta \equiv L/L_{BB}$ is fixed by energy

conservation. The incident intensity is thus $I_{\nu}^{inc} = \delta B_{\nu}(T_{BB})$. For all models reported here a rough fit to the observed continuum shape is achieved with $T_{BB} = 5,000$ K. The dilution factor depends both on the selection of R_{ph} and on the extent of ^{56}Co mixing. The latter determines the actual luminosity at the photosphere. Many weak lines combine to form a quasi-continuum in the observer's frame which is smoothed further by electron scattering. Electron scattering will both broaden the observed line profile and decrease the luminosity at the line centre, because of the differential expansion of the atmosphere¹⁸. Our photosphere effectively smooths the detailed line spectrum that would be emitted from the material below R_{ph} , replacing it with a continuous spectrum. The core material of the atmosphere has a higher density and metal content (metallicity) relative to the envelope (see below); therefore, the electron density is relatively large and many weak metal lines are formed in the core. Our lower boundary condition most reasonably represents the natural conditions when it is placed within or immediately above the core.

This study is confined to hydrodynamic models and composition-mixing schemes for supernova 1987A that have been presented already in the literature. The canonical model comprises a $6-M_{\odot}$ core, composed chiefly of helium and oxygen, and a $10-M_{\odot}$ hydrogen-rich envelope. The inner $1.4 M_{\odot}$ forms a neutron star. Three composition-mixing variations for this model are shown in Fig. 1. The variations correspond to models 10H (ref. 5) (composition I), 10HM (ref. 9) (composition II) and 10HMM (ref. 11) (composition III), which were provided by S. Woosley (private communication). The increased composition mixing represents successive improvements to the theoretical light curves and γ - and X-ray spectra reported in refs 5, 9 and 11.

We present results that are valid at 200 days after the supernova event, when the deeper layers may be observable. The placement of the lower boundary, R_{ph} , and the distribution of ^{56}Co are the only free parameters of the models. The total mass of radioactive ^{56}Co in all models is $0.075 M_{\odot}$. Theoretical spectra for the 'standard' Type II-supernova model, consisting of a stratified core composition and with the ^{56}Co confined to the central region, are not consistent with the observed spectra of SN1987A. We therefore concentrate on models that incorporate various degrees of mixing.

Spectra of several models computed using composition III with different photospheric positions are shown in Fig. 2. For these calculations, the radioactive cobalt is distributed with constant mass fraction out to a mass cut of $14 M_{\odot}$. The best fit to the spectral line strengths and shapes corresponds to $R_{ph} = 3.0 \times 10^{15}$ cm, a radius just outside the 'core'. The best fit to the continuum flux also occurs for this value of R_{ph} , although this result depends to some extent on the ^{56}Co distribution as well (see below). This same value of R_{ph} also produces the best results for models using compositions I and II. The spikes seen in the lower two spectra of Fig. 2 are accentuated in those models that feature increased stratification. These spectral features arise from the increased density in the low-velocity core region and are stronger for higher metallicity. Most of the metal lines for models using compositions I and II are too strong if the core regions are left exposed. We conclude that broad-band opacity effects become important below the core/envelope boundary.

For each of the three compositions we have calculated spectra assuming that the cobalt is distributed below a mass cut M_c of 5, 10 and $14 M_{\odot}$ and assuming that there is a constant mass fraction of ^{56}Co below the cut. These mass cuts correspond to maximum ^{56}Co velocities of $1.3, 2.5$ and 3.8×10^3 km s⁻¹ respectively. The resulting spectra for these ^{56}Co mixing variations, using composition III, are shown in Fig. 3, for which the value $R_{ph} = 3.0 \times 10^{15}$ cm is adopted. For $M_c < 10 M_{\odot}$ the hydrogen lines are too weak and all the spectral lines appear too narrow, as can be seen dramatically in the Ca II infrared triplet. The

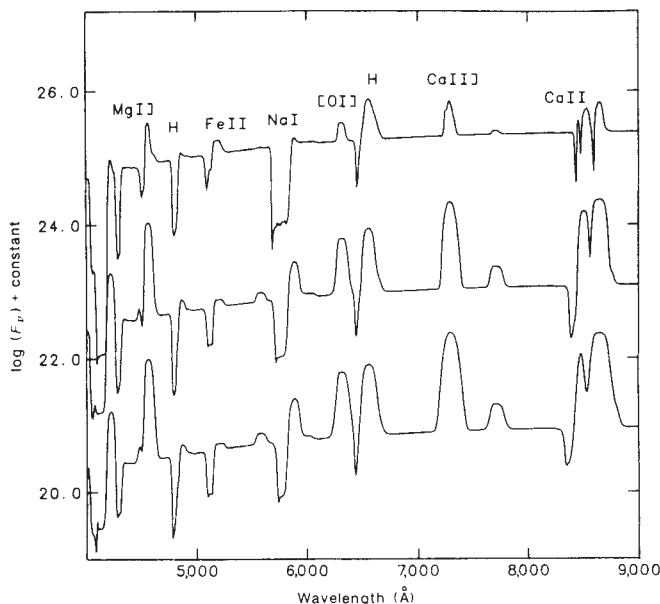


Fig. 3 Spectra for composition III at 200 days for different ^{56}Co mass distributions. The total ^{56}Co mass in all cases is $0.075 M_{\odot}$. In all cases, the photosphere is located at radius $R_{\text{ph}} = 3.0 \times 10^{15}$ cm and the assumed temperature $T_{\text{BB}} = 5,000$ K. From bottom to top the ^{56}Co is distributed out to a mass point M_c of 14, 10 and $5 M_{\odot}$. For clarity the spectra have been shifted relative to each other by two logarithmic units on the vertical axis. Note the increased continuum flux (most visible at the red edge of the spectra) for smaller M_c , which is due to the increased total luminosity from the opaque region. From bottom to top the luminosity at the photosphere is $5.7, 7.9$ and $15.6 \times 10^{40} \text{ erg s}^{-1}$.

same results occur for the other composition-mixing models with this value of M_c . The strength of the metal lines is satisfactory for composition III with $M_c = 5 M_{\odot}$, because of the large mass of heavy elements mixed into the envelope in this model. The metal lines are not strong enough for compositions I and II when such a low value of M_c is used. We conclude that the best fit to the observed spectra occurs for $M_c \geq 10 M_{\odot}$. Several studies⁹⁻¹¹ impose an outwardly decreasing mass fraction for the ^{56}Co distribution. We find that the optical spectrum is not sensitive enough to the details of the ^{56}Co distribution to differentiate between models with a gradient in ^{56}Co mass fraction and models with a constant mass fraction, provided that the decay energy is deposited throughout a major fraction of the ejecta. Our models require that the radioactive energy be deposited throughout a substantial fraction of the envelope; however, the cobalt could remain confined to the core if some mechanism exists for transporting the energy outward.

A comparison between the observed spectrum (M. Dopita, private communication) and the best results from the three mixing scenarios is presented in Fig. 4. The photospheric radius $R_{\text{ph}} = 3.0 \times 10^{15}$ cm (mass point $4.6 M_{\odot}$) for all models, so that only envelope material contributes to the lines, and $M_c = 14 M_{\odot}$. The lower metallicity of the unmixed model (composition I) provides the best overall fit, particularly at the red wavelengths, but all three models provide qualitative agreement with the observed spectrum. We cannot rule out any of these three models on the basis of these spectra, but composition I is preferred. The Ca II infrared triplet is too strong in all models by a factor of two or three. The Ca II line strengthens as the metallicity increases and becomes too wide for composition III. As electron scattering tends to broaden the lines, we conclude that the fit for this line is poor for composition III. The strength of H α is best for composition II although the red wing is weak in all models. The [O I] 6,300-Å line is accurately represented by the composition I model, as is the [O I] 6,300/[O I] 5,577 ratio. The Na I D-emission wing is too strong for all models by a factor

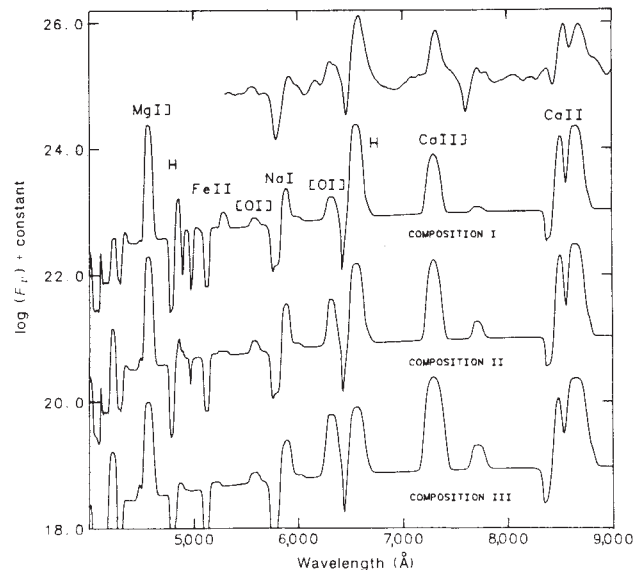


Fig. 4 Comparison of three mixed-composition models for observations at 200 days. From top to bottom the spectra are the observed spectrum (M. Dopita, personal communication) and models for compositions I, II and III. All models use $R_{\text{ph}} = 3.0 \times 10^{15}$ cm and $M_c = 14 M_{\odot}$. For clarity the spectra are shifted vertically as before.

of two to three. At wavelengths shorter than those of the observed spectrum is the Mg I] 4,571-Å line. This line is very strong in all our models, including the many not discussed here. We note that this line is predicted to be strong by other models as well³ but is not observed until much later times.

Based on these results, we conclude that (1) a good qualitative fit to the optical spectrum of SN1987A is obtained from the hydrodynamic models at 200 days if the lower boundary is placed just above the core; (2) the lack of observed narrow-line cores suggests that the dense slow-moving core region does not contribute substantially to the emission lines at 200 days; (3) a considerable contribution to the observed optical emission lines comes from the heavy elements in the envelope even if no composition mixing is invoked; (4) the radioactive decay energy must be distributed throughout a major fraction of the mass of the star ($M_c > 10 M_{\odot}$) to be consistent with the observed spectrum; and (5) there is evidence for strong broad-band opacity at late times that is responsible for producing P-Cygni line profiles, as represented by our lower boundary.

Based on the limitations of computations used in this study, we further conclude that a large number of realistic model atoms, providing many spectral lines, is required to improve accuracy and that continuum-transfer effects should be included in future studies of this kind.

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Rapid variability of extragalactic radio sources

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Since its discovery more than 20 years ago^{1,2}, variability of extragalactic radio sources on timescales of weeks to years has been the subject of many investigations. Little is known, however, about radio variability of extragalactic sources on timescales of less than a few days. Variations on timescales of hours—mostly at millimetre wavelengths—have been reported for several sources³⁻⁶. Heeschen *et al.*⁷ have found variability with amplitudes of up to 12% in less than 1 day at 11-cm wavelength in a sample of northern ($\delta \geq 60^\circ$) compact sources. We have continued these observations at wavelengths of 6 and 11 cm using the 100-m telescope of the Max-Planck-Institut für Radioastronomie and report here the results for two sources. The quasar QSO0917+62 showed variations with amplitudes of up to 23% in ~24 hours, which were correlated at the two wavelengths; in the BL Lac object 0716+71 we found variations with amplitudes of 7–11%. We discuss intrinsic effects, gravitational lensing and scattering in the interstellar medium as possible explanations for rapid radio variability.

The observations were made in two sessions in 1988: 31 March to 5 April (112 hours, 6- and 11-cm observations made almost simultaneously) and 15–20 June (104 hours, 6 cm). For the 11-cm measurements we used a correlation receiver (cold-load reference) with a bandwidth of 80 MHz and a system temperature of 55 K; the 6-cm measurements were performed using a dual-beam correlation receiver with a bandwidth of 500 MHz and a system temperature of 65 K. Both receivers have circularly polarized feeds and are mounted in the secondary focus of the 100-m telescope. For each observing epoch, our sample included nine flat-spectrum compact sources (spectral index $\alpha_{11}^{\nu} \geq -0.5$; flux density $S \propto \nu^\alpha$) and seven or eight steep-spectrum reference sources of similar flux densities. The observations consisted of cross scans in azimuth and elevation through the source position, thus allowing for accurate correction of pointing errors (see Heeschen *et al.*⁷ for details of the observations and data-reduction procedures, including corrections for gain and extinction). In addition, the influence of weak confusing sources was estimated from the difference of scans in azimuth and elevation as a function of parallactic angle; a corresponding correction was applied to the 11-cm data of six sources. The sum of all corrections applied to the raw data never exceeded 2%. For all sources we calculated a percentage fluctuation index $u = 100 \sigma / \langle S \rangle$, where $\langle S \rangle$ is the mean flux density of the source in the observing session and σ is the standard deviation of single measurements. The errors are dominated by systematic errors (that is, proportional to S); their level is given by the mean fluctuation index for steep-spectrum sources, which is 0.27% at 11 cm and 0.35% at 6 cm wavelength. (The value at 11 cm wavelength reproduces

exactly that of Heeschen *et al.*⁷.) As none of the included steep-spectrum sources showed peak deviations of more than 1% from their mean flux densities (see for example 0951+69 in Fig. 1a), instrumental artefacts above this level can be excluded.

Of all observed sources, the quasar 0917+62 showed the largest variability during both sessions: 22% (peak-to-peak) at 11 cm and 14% at 6 cm in the March/April run, and 23% at 6 cm in June. In the figures, normalized variations $R = 100 (S/\langle S \rangle - 1)$ are plotted against time. The fastest 'events' occurred at 6 cm wavelength in March/April; our ~75-min sampling after JD 2447255.4 reveals fine structure in the light curve on a scale of ~4 hours (Fig. 1b). The variations for the 11-cm data (Fig. 1a) are similar but much smoother; however, they show a remarkable discontinuity of the slope at JD 2447256.0, at about the same time as the secondary maximum at 6 cm. Two similar discontinuities are apparent in the 6-cm data for June (Fig. 1c), as well as a spike-like feature at JD 2447331.1, the amplitude of which is significantly above the uncertainty level. For the single observations of 0917+62 in March/April, the 6-cm variations are faster than the 11-cm variations by a factor of about three. We also note that, from visual inspection of the light curves, there is the suggestion of a time lag of ~1 hour near the minimum at JD 2447255.6, with the 11-cm data lagging behind the 6-cm data (Fig. 1a and b). A formal cross-correlation analysis of the full data set gives a time lag of 0.5 ± 1 hours. In 1985, 0917+62 was observed twice at 11 cm wavelength⁷; it was variable in both sessions, with amplitudes of 3.4% and 8% respectively.

In contrast to 0917+62, the BL Lac object 0716+71 showed quite different light curves at 6 cm and 11 cm in the March/April dual-frequency run; it is not obvious how these two curves are related to each other. This could imply a time lag longer than the length of our observing period, or it may be that the variations at 6 and 11 cm are indeed unrelated. The light curves of 0716+71 also show significant fine structure on timescales of a few hours, which were not adequately sampled (Fig. 1d). In each of the four sessions during which it was observed since 1985, 0716+71 showed a remarkable consistency of behaviour; its variability was always in the narrow range of 7–11%.

We note that rapid radio variability seems to be a quite common phenomenon. It was observed in 6 out of 15 sources by Heeschen *et al.*⁷; three of the sources are classified as quasars and three as BL Lac objects. These sources had been selected on the basis merely of their high declination, flat spectrum, and high flux density. Furthermore, the occurrence of a similar effect—a 7% decrease of the 6-cm flux density in slightly more than 1 hour—in OJ287 (ref. 6) indicates that it is not limited to the north-polar region. On the basis of up to five observing periods now available for several sources, it seems that rapid variability is not distributed randomly among compact sources, but rather that it appears quite regularly in the same sources each time they are observed. Thus this phenomenon is connected either to certain characteristics of the sources (besides compactness, which seems to be a necessary but not sufficient condition) or to their positions in the sky. As is already apparent from the two cases of 0716+71 and 0917+62, the detailed wavelength dependence of the variations—including cross-correlation and possible time lags—is different in the observed sources, but the amplitudes at 6 and 11 cm wavelength are similar.

At least three different mechanisms could explain the observed variability: intrinsic effects, gravitational (micro-) lensing and refraction in the interstellar medium (scintillation).

Although the variability on somewhat longer timescales observed at high frequencies, in 3C279 and OJ287 for example, is connected to variations in the degree and angle of polarization and is therefore generally interpreted as being intrinsic^{8,9}, it is difficult to attribute variability as rapid as reported here to intrinsic effects. Standard light-travel-time arguments would lead to extremely small source diameters and therefore to brightness temperatures as high as 2×10^{17} to 7×10^{18} K (under the

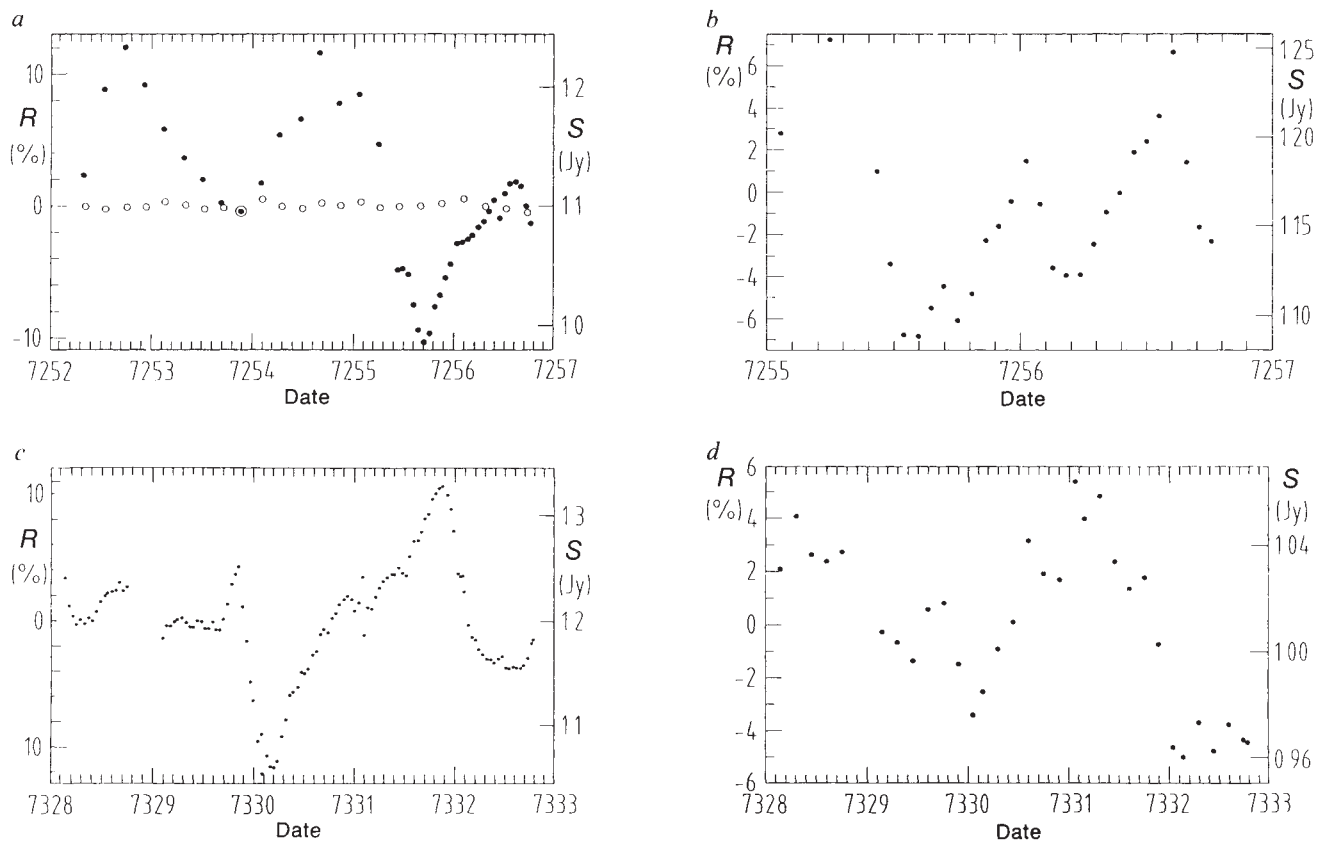


Fig. 1 *a*, Flux density (S) of 0917+62 (filled circles) and the steep spectrum reference source 0951+69 (open circles) at 11 cm wavelength in March/April 1988 against date (JD-2440000). Normalized variations R are defined as $100 (S/\langle S \rangle - 1)$. The data for the steep spectrum source indicate the total measurement errors. The absolute scale applies to 0917+62. *b*, Flux density of 0917+62 at 6 cm wavelength, March/April 1988. *c*, Flux density of 0917+62 at 6 cm wavelength, June 1988. *d*, Flux density of 0716+71 at 6 cm wavelength, June 1988.

assumption that the unknown redshift of 0917+62 lies in the range 0.1–1), so that for incoherent synchrotron radiation, Doppler boosting factors of 50–200 would be needed to meet the Compton limit of about 10^{12} K (ref. 7). The same argument applies to scattering by material within the quasar or at cosmological distances: either the source of radiation must be very small, or the apparent relative transverse velocity must exceed $\sim 100c$. Coherent mechanisms could also produce fast intrinsic variability, but no convincing evidence for those processes has been found so far. Normally, intrinsic variability is larger at shorter wavelengths, in contrast to the variations observed here, but with the present limited understanding of the relevant physical processes we cannot rule out intrinsic effects as a possible explanation.

If the radiation from a distant quasar passes through an intervening galaxy, one must take into account the relativistic light deflection not only by the whole galaxy but also by individual stars¹⁰; the combined effects of many stars lead to a distribution of caustics in the source plane. Calculated model light curves look qualitatively very much like those shown here: they show similar discontinuities of the slope, which reflect the convolution of the moving source structure with the distribution of caustics^{11,12}; the details depend on the models adopted for the lensing galaxy and the source structure. Gravitational lensing is sensitive to wavelength only through the wavelength dependence of source size and shape, so that it can explain the observed similar variations at 6 cm and 11 cm. Nevertheless, of the above mentioned explanations, gravitational micro-lensing seems the least probable, because we could not find, on the POSS plates, any obvious lens candidates in the fields of 0716+71 and 0917+62. Furthermore, to produce a noticeable effect, the source size cannot be much larger than the variability time-scale multiplied by the velocity of light. This leads to about the

same source size as required by intrinsic explanations and thus encounters the same difficulties.

Scattering by the interstellar medium is believed to cause low-frequency variability^{13,14}, as well as flickering at ~ 10 cm wavelength on timescales of several days to weeks^{15,16}. Unusual features in the light curves of compact extragalactic sources sampled once per day¹⁷ are also attributed to scattering by compact structures in the interstellar medium. In the single-scale model, the timescale is simply given by the spatial scale divided by the relative velocity. For galactic velocities of 200–300 km s⁻¹ or less, the durations of about a day for single events then imply that the scattering density fluctuations cannot be much larger than ~ 0.1 AU. The electron density n_e of single scattering blobs can be estimated from $\theta \approx (2\pi)^{-1} r_e \lambda^2 n_e$, where θ is the refraction angle, r_e the classical electron radius and λ the wavelength of the observation. For $\theta \approx 1$ milliarcsec this yields $n_e \approx 10^3$ cm⁻³ and thus a mass of $\sim 2 \times 10^{16}$ g. At a distance of 1 kpc a scatterer of diameter 0.1 AU covers $\sim 10^{-18}$ sr, so a total mass of $\sim 2 \times 10^{34}$ g, or $10 M_\odot$, is needed to reproduce the observed scattering probability. Although very little is known about the structure of the interstellar medium on scales as small as those involved here, and although electron densities of 10^3 seem quite large, the extreme scattering events¹⁷ give strong evidence for the presence of such clouds. Because of the small timescales present in our data, refractive interstellar scintillation in a medium with a power-law wavenumber spectrum and the standard parameters given by Rickett¹⁸ gives no consistent results, unless the distance to the scattering screen is reduced to ~ 50 pc. Normally scattering by the interstellar medium is expected to have higher amplitude at lower frequencies, but again the dependence of source size on wavelength may lead to equal amplitudes at different frequencies^{19,20}, or even to higher amplitudes at higher frequencies, as observed in the extreme scattering events¹⁷. In this framework,

caustics may also be relevant²¹, thus providing an explanation of the observed shapes of the light curves as mentioned above.

Because our sample sources were selected on the basis of their high declination, they all lie in the same region of the sky, which happens to be close to the galactic loop III. It seems possible that the observed variability is connected to this old supernova remnant⁷ or to the high-velocity features present in the same area^{22,23}. This interpretation is not completely satisfactory, however, because OJ287 shows a similar behaviour⁶, but is not located near any known feature resembling the galactic loops.

It is obvious that, irrespective of its reason, variability with amplitudes of several per cent and timescales of hours causes severe problems to aperture-synthesis observations, whose dynamic range may be limited by fluctuations in the source brightness as small as 1% (refs 7, 19). Thus for observers who wish to map low-brightness features in strong sources, to look for weak images in gravitational-lens systems or to investigate clustering of radio sources, it is very important to know in detail the characteristics of rapid variability.

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Slowly moving thermal features on Jupiter

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Obtaining measurements of winds on the jovian planets—Jupiter, Saturn and Uranus—at depths other than the level of the observed visible clouds has long been a problem preventing an understanding of the dynamics of these deep atmospheres^{1,2}. Measurements of horizontal thermal structure on Jupiter suggest that the observed zonal (west–east) jet system decays slowly above the visible clouds, persisting over many pressure-scale heights³. The ubiquitous visible cloud cover, believed to be composed of ammonia ice crystals lying somewhere between the 700-mbar and 300-mbar pressure levels⁴, prevents direct sensing below these levels, so the depth of the wind

system remains unknown. Here we report that maps of upper troposphere temperatures derived from Voyager 1 and Voyager 2 infrared interferometer spectrometer (IRIS) spectra show large-scale features which move very slowly; the motion is very different from the inferred local fluid velocity. We suggest that these features may be indicative of a deeply-rooted fluid dynamical regime beneath the surface meteorology, although other explanations are possible.

The Voyager IRIS instruments obtained measurements of the thermal emission of Jupiter over the spectral range 4–55 μm (ref. 5). During the encounters with Jupiter, at a distance of about 3×10^6 km before and after closest approach, the instruments were used to map the planet globally by scanning from north to south along the sub-spacecraft meridian for one rotation of the planet, to produce north–south maps with a spatial resolution of about 10 degrees of longitude at the sub-spacecraft point. Between these sequences, higher-spatial-resolution observations of selected features were obtained. The spectra have previously been inverted to deduce the temperatures at the 270-mbar and 150-mbar pressure levels, the ammonia abundance, and cloud optical depths at 5 and 45 μm (refs 3, 6). Cylindrical projection maps, in the form of digital images, have been created for each of these quantities from the inbound and outbound north–south mapping sequences obtained by Voyager 1 and the inbound map from Voyager 2. Zonal-mean motions as a function of latitude have been evaluated from time-lapse map pairs by using a line-by-line correlation technique previously applied to Voyager imaging mosaics⁷. The velocity resolution for the time interval between the inbound and outbound maps from Voyager 1 is 30 m s^{-1} and for the time interval between Voyager 1 and Voyager 2 is 1 m s^{-1} , based on the IRIS instrument beam size.

Figure 1 displays the correlations between the Voyager 1 inbound and outbound north–south maps of temperature at a pressure of 270 mbar, and of cloud optical depth at 45 μm . The well defined and continuous line of maxima shows that meaningful correlations have been achieved, and this line defines a zonal displacement profile. The most striking difference between the images is the absence, in the temperature correlation image, of the broad region of positive displacement near the equator. Figure 2 shows the displacements corresponding to the maxima from Fig. 1, in units of zonal wind, and the linear-correlation coefficients. Based on a sampling of one observation point every ten degrees, the probability of achieving a correlation coefficient of 0.4 or better for one longitude strip from a random sample of uncorrelated observations is less than 2%. Plotted along with the IRIS results is a zonal-mean-wind profile derived from Voyager imaging-camera data that has been degraded to the same spatial resolution as the IRIS north–south maps. The wind profile from the infrared–optical-depth maps is in good agreement with the visible-imaging profile to within the spatial and velocity resolution limits. In the equatorial region a broad, strong prograde jet exists and is much wider than the spatial resolution of the IRIS beam. Jets observed at higher latitudes with the imaging camera are too narrow to be resolved by the IRIS instrument. Although wide enough, the strong jet at 24°N is not detected because of the lack of longitudinal structure. The results in Fig. 2 indicate that the infrared data is capable of recovering the low-latitude cloud motion field but that the dominant thermal structures do not participate in its motion.

Longitudinal scans from both the ingoing and the outgoing Voyager 1 maps of the 270-mbar temperature field and cloud optical depths at the equator are plotted in Fig. 3. The outgoing scans have been displaced in longitude by the correlation results to compensate for zonal drift and to facilitate comparison of structures. The largest-amplitude thermal structures at 270° , 210° and 60° longitude clearly show the features determining the correlation result. Some weak features do not coincide for the two scans and may drift at the higher rate of cloud features, but enough agreement exists such that identifications can be

Fig. 1 Correlation images for temperature at 270 mbar (top) and aerosol optical depth at 45 μm (bottom) from Voyager 1 inbound and outbound north-south maps. The value of the correlation function is represented by variations in colour and is displayed for each line of planetographic latitude as a function of eastward longitudinal displacement (degrees) between the lines of the inbound and outbound maps. A positive shift of 30° corresponds to a prograde velocity of $\sim 100 \text{ m s}^{-1}$ at the equator.

Method. Cylindrical-projection digital images of the north-south maps used in the correlations were produced by first subtracting the zonal average of the quantity at the latitude of the observation from each measurement. Then the value of the image at each pixel was expressed as a sum of all observations within a given range weighted by a modified gaussian function of the distance between the pixel and the original observation point. This procedure is necessary because the IRIS is a non-imaging instrument. A correlation image is produced by evaluating the correlation function for corresponding lines of a map pair as a function of longitudinal displacement between the lines. For each line of latitude the maximum and minimum correlation-function values are assigned to the corresponding ends of the colour scale.

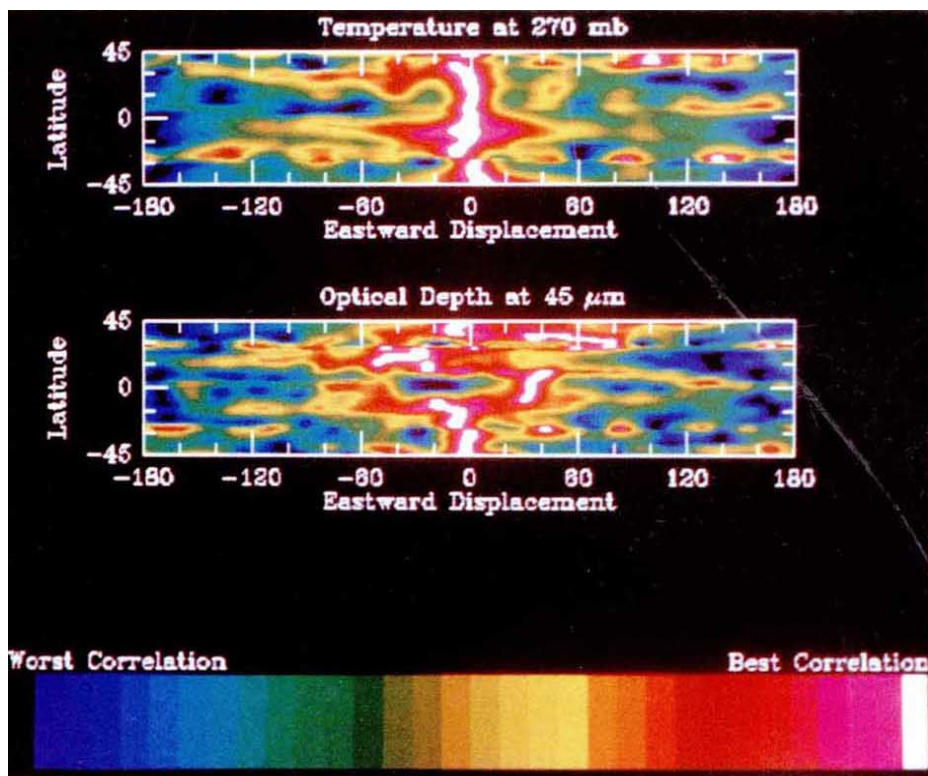
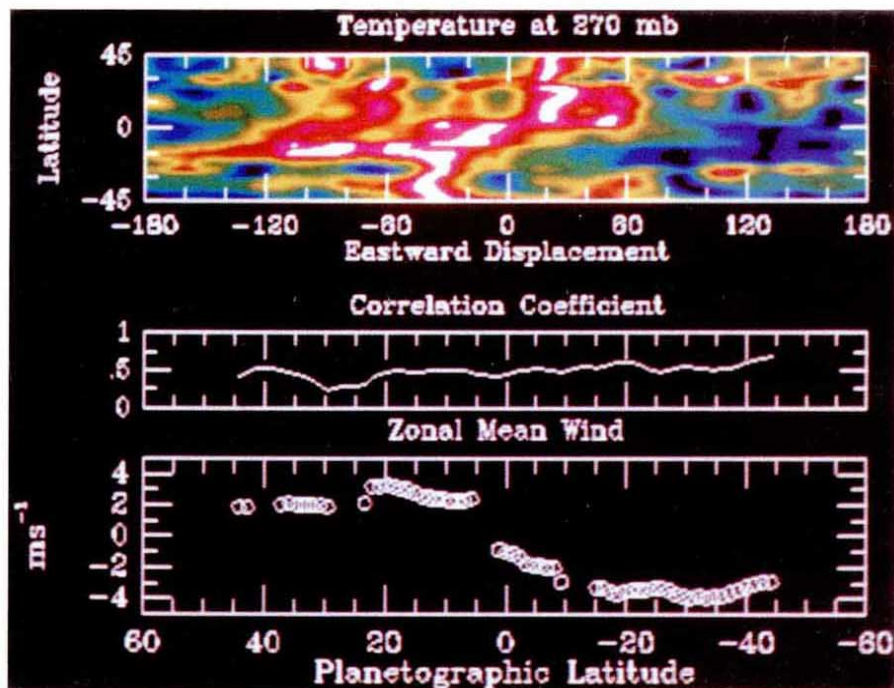


Fig. 4 Correlation results for temperature data (270-mbar level) from Voyager 1 and Voyager 2 inbound maps. Top, Correlation image. Bottom, Zonal velocity versus latitude from the line of maxima in the correlation image. Middle, Linear correlation coefficients from the maxima. See legends to Figs 1 and 2 for further explanation.



made. The small thermal contrasts of the dominant structures are well above the formal noise level of the IRIS. The cloud-optical-depth scans show some structural agreement, although significant changes have occurred; temporal variability in the atmosphere and zonal variation in the wind can lead to such changes. The linear-correlation-coefficient values provide a quantitative measure of the agreement between pairs of scans. The lower correlation coefficients of the optical depths as well as the longitudinal scans in Fig. 3 show that the horizontal cloud structure is more variable than the thermal structure. This result is consistent with Jupiter's pronounced visible variability, which

exists in spite of the overall stability of the jet system^{1,2}.

Further evidence of slowly moving thermal features can be obtained in at least one latitude range. During the closest approach by Voyager 1, Jupiter was longitudinally well sampled between 5° and 15° N at high resolution (about 2 degrees of longitude at the sub-spacecraft point). Fitting a Fourier series to the thermal data retrieved from this sequence, as well as to the corresponding original values in the inbound and outbound north-south maps (that is, before they were made into images), shows a strong component at a wave mode of 9. A least-squares fit of the phase velocity of this wave between all three data sets

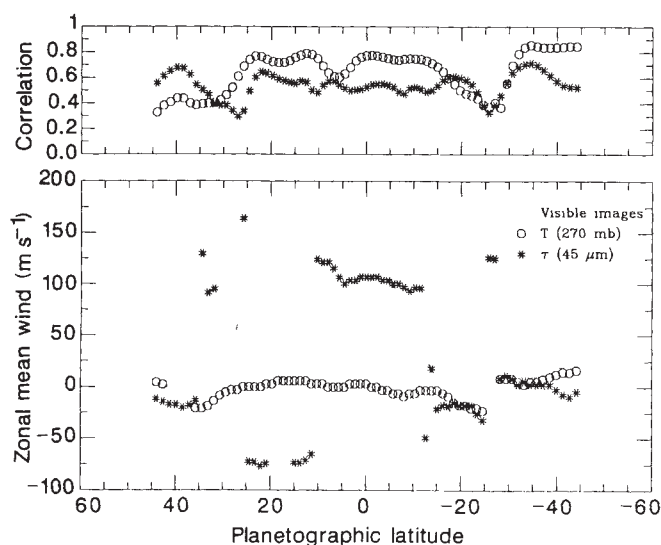


Fig. 2 Zonal velocity versus latitude from temperature (at 270-mbar level), optical depth (at $45\text{ }\mu\text{m}$) and Voyager 2 violet-image correlations (bottom panel only) (dashed line). The velocities are derived from Fig. 1 by expressing, in units of zonal wind, the displacements associated with the correlation-function maxima. Some missing points are evident at selected latitudes, where spurious correlations occur. Linear-correlation coefficients of the maxima are displayed in the top panel for the infrared results. The visible image velocities are obtained from Voyager 2 violet-image mosaics and have been degraded to the spatial resolution of the IRIS.

yields a value of $10.5 \pm 12.4\text{ m s}^{-1}$ (recall that the dynamically important quantity is the phase velocity relative to the mean flow, which, for this range of the total phase velocity, is large and negative).

The high correlation coefficients for the temperature data from Voyager 1 suggest that correlation with the Voyager 2 thermal map might be possible. Moreover, the upper bound on the drift of features provided by the Voyager 1 results indicates that the thermal structures would drift by significantly less than one planetary circumference during the time interval between the Voyager 1 and Voyager 2 encounters (~ 4 months). Thus, velocities could be obtained unambiguously from the correlations. Figure 4 displays the results of such a correlation. The line of maxima is remarkably continuous and well defined, and suggests that meaningful correlations have been achieved, although the maxima are broader, and the correlation coefficients somewhat lower, than the Voyager 1 results. The line of maxima shows a striking antisymmetry about the equator with an amplitude of $\sim 2\text{--}4\text{ m s}^{-1}$. The velocity corresponding to the maximum at 23° S latitude agrees with the velocity of the Great Red Spot inferred from imaging data² to within the formal velocity resolution based on the IRIS beam size ($\sim 1\text{ m s}^{-1}$). A more conservative estimate of the velocity resolution is the width of the half-maximum point of the correlation, which yields a resolution of $\pm 5\text{ m s}^{-1}$; this resolution is still significantly better than that of the Voyager 1 map correlation. In Fig. 2 the longitudinal scans of the equator show structures at longitudes 270° , 200° and 70° which match the Voyager 1 features, although the matches are certainly not as clear as those in the comparison of Voyager 1 inbound and outbound maps.

The zonal winds in the upper troposphere of Jupiter are believed to be comparable to the winds seen in the clouds, because the vertical zonal-wind shear at these levels is quite small³. Although thermal perturbations that drift with the tropospheric currents undoubtedly exist, they are apparently of such small amplitude or horizontal scale that the IRIS beam does not resolve them. The thermal features described here drift slowly with respect to the bulk rotation of the planet as inferred from radio observations¹ and far more slowly than the local aerosol velocity ($\sim 100\text{ m s}^{-1}$). If the thermal perturbations producing these structures are excited in a region where the fluid velocity is comparable to the observed equatorial jet, then the observed motion of the structures would represent a large negative phase velocity with respect to the fluid, and this phase drift would need to have a magnitude sufficient to nearly cancel the fluid motion. Because such nearly exact cancellation of the

equatorial jet velocity by the phase velocity seems improbable, we believe that the slowly moving thermal features must be excited in a region of slow or no motion. The excitation could result from the influence of dynamical structures in a slowly moving deep atmospheric circulation, from horizontal propagation from slowly moving features at higher latitudes (such as the Great Red Spot), or from upper-atmospheric or magnetospheric features moving with the planetary rotation. We consider the last possibility to be unlikely because of the relatively small energy content of the upper atmosphere. Horizontal propagation may be possible but would need to extend across large horizontal zonal-wind shears. We conclude that the thermal features described here probably represent the influence of slowly moving dynamical structures below the visible clouds on Jupiter.

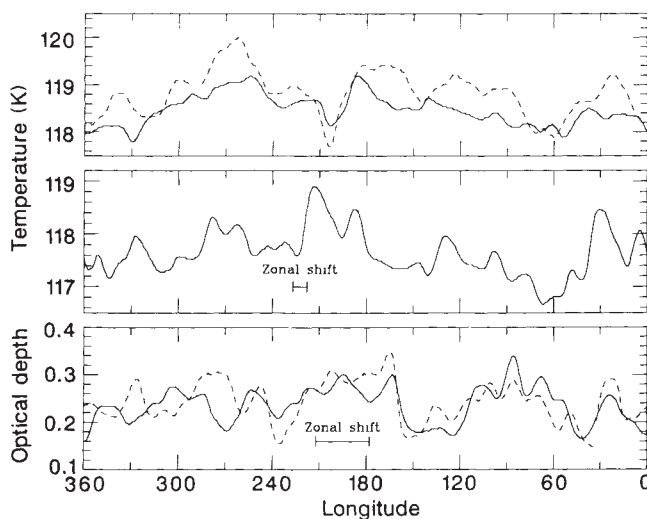


Fig. 3 Longitudinal scans of the equator from Voyager IRIS maps of temperature and optical depth. Top, Scans of temperature from Voyager 1 inbound (solid line) and outbound (dashed line) maps. Middle, Scan from Voyager 2 inbound map. To facilitate comparison of thermal structures, the Voyager 2 scan has been displaced to compensate for zonal drift using the correlation displacement; the size of the shift is displayed. Bottom, Scans of cloud optical depth from Voyager 1 inbound (solid line) and outbound (dashed line) maps. The outgoing map scan has been displaced to compensate for zonal drift using the displacement from the correlation-function maximum in Fig. 2; the size of the shift is displayed at the bottom of the panel.

Previous efforts at inferring the dynamics and structure below the clouds have used wave signatures or flow fields in the visible cloud layers combined with modelling to draw inferences about deep physical properties^{9,10}. However, such inferences are somewhat ambiguous and limited to particular regions. These techniques are similar in spirit to seismic studies of the Earth's internal structure and to acoustic-gravity-wave sounding of the Sun¹¹. The thermal anomalies reported here may provide a new means of probing Jupiter.

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Direct ozone depletion in springtime Antarctic lower stratospheric clouds

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It is now generally accepted that springtime Antarctic ozone depletion in the atmosphere between 17 and 22 km is related to the presence of ClO produced through heterogeneous chemistry on the surface of polar stratospheric cloud particles^{1,2}. Below 17 km, however, where ozone depletion also occurs³, a causal link with ClO has not been established because of apparently low ClO values. Vertical motions, associated with mountain lee waves and the formation of nacreous clouds, also seemingly cannot explain observed ozone depletion⁴. Here we present balloon measurements of ozone and cloud particles in the lower stratosphere (10–17 km) at McMurdo Station (78°N), Antarctica, on 9 September 1988 which indicate that the cloud particles are directly involved in ozone depletion in this region. The reductions in ozone appear to be correlated with high concentrations of smaller particles, of about 0.2 micrometre radius, which dominate the surface area density in the cloud. The observations suggest a high degree of spatial inhomogeneity of free chlorine, associated with lower stratospheric clouds, and are important in understanding ozone depletion in this region.

Instruments used in the balloon flights included a temperature and electrochemical ozone sensor of high vertical resolution⁵, and an individual particle optical counter similar to types used previously in Antarctica⁶ but with an air-sample flow rate about 13 times higher ($\sim 200 \text{ cm}^3 \text{ s}^{-1}$) so as to resolve the low concentrations ($\sim 10^{-3} \text{ cm}^{-3}$) associated with ice crystal formation. The latter instrument measured in seven size ranges, that is, for radii $r \geq 0.20, 0.25, 0.30, 1.0, 2.0, 3.0$ and $5.0 \mu\text{m}$, for spherical particles or for slightly non-spherical particles of equivalent volume⁵.

Although most flights reached the 30-km altitude region, we concentrate here on the 10–20-km region investigated on 9 September 1988, for which Fig. 1 shows the temperature and ozone data observed. Also shown for comparison are data obtained on 4 September, which are typical of profiles observed before this time. On 9 September the region between 10 and

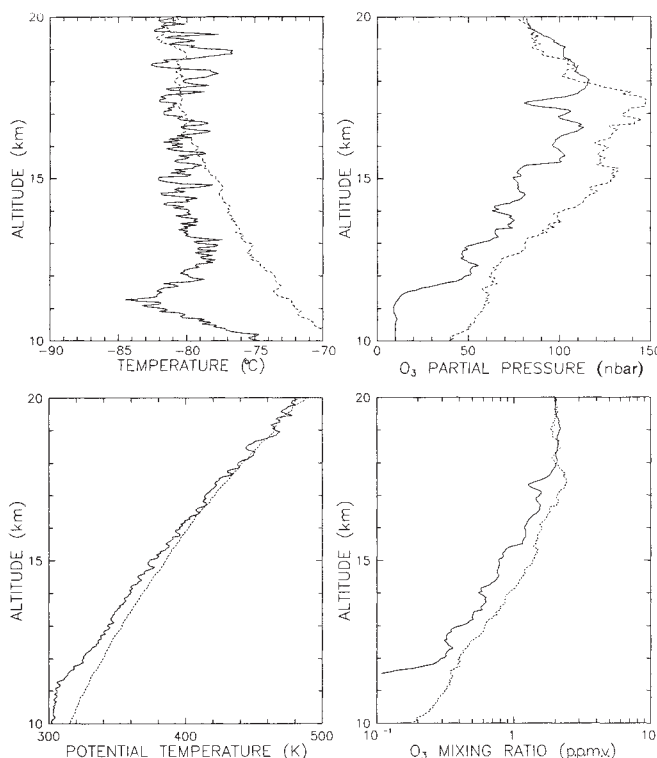


Fig. 1 A comparison of temperature, ozone partial pressure, potential temperature and ozone mixing-ratio profiles measured between 10 and 20 km at McMurdo Station on 4 (dashed line) and 9 (solid line) September 1988.

~ 11.5 km (an unusually high tropopause) is well mixed and appears to be associated with upward motion; it was in this region that particles with $r \geq 5.0 \mu\text{m}$, presumably comprising water ice crystals, were observed. Above this region, the temperature data indicate a high degree of wave-like stratification with a wavelength of a few hundred metres and an apparent growth in wave amplitude with altitude. The growth rate is consistent with non-dissipative wave propagation, which would double the amplitude between 12 and 20 km.

Reductions in ozone partial pressure at about 12.7, 14.1, 15, 16 and 17.2 km appear similar to those arising from vertical motions, but this is not borne out by the temperature data, as indicated by the potential temperature and ozone mixing-ratio profiles in Fig. 1. Although there exist regions of constant ozone mixing ratio on 9 September, these appear to be unrelated to vertical mixing, because the potential temperature does not show corresponding regions of constancy, as observed in Antarctic mountain-lee-wave events⁴. The region of ice crystal formation on 9 September (10–11.5 km) does show constant potential temperature, associated with cooling of the air parcel by an adiabatic lifting process. The low ozone concentrations in this region are typical of upper-tropospheric values.

The particle measurements made on 9 September are central to an interpretation of the ozone data. Figure 2 shows the 10–20-km profiles of three size ranges, $r \geq 0.20, 1.0$ and $5.0 \mu\text{m}$, for 9 September, compared with data obtained with an identical instrument five days earlier. The data for $r \geq 0.20 \mu\text{m}$ on 4 September indicate a normal sulphate layer and normal absence of larger particles, as seen in 1986 and 1987⁵, except at ~ 18 km, where there appears to be a slightly enhanced small-particle layer. In contrast, the 9 September profiles are highly enhanced between 11.5 and 17.2 km and between 19 and 20 km; the concentration for $r \geq 0.20 \mu\text{m}$ is about 10 times greater than normal at ~ 12 km. The observations were verified during parachute descent.

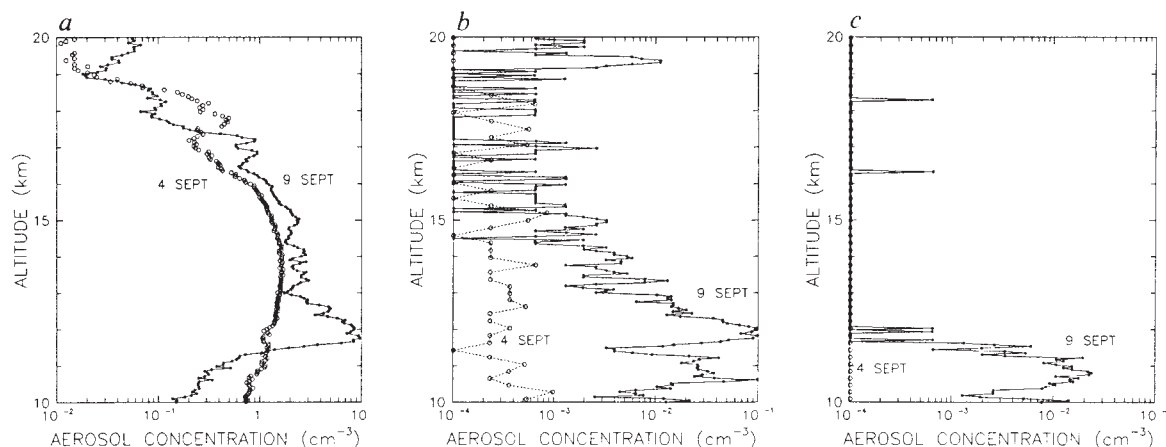


Fig. 2 Aerosol profiles for radii greater than 0.20 (a), 1.0 (b) and 5.0 (c) μm measured between 10 and 20 km at McMurdo Station on 9 September 1988 compared with those measured under non-enhanced conditions on 4 September.

The total aerosol concentration, as observed with a condensation-nuclei ($r \geq 0.01 \mu\text{m}$) counter on 6 and 14 September, was $\sim 20 \text{ cm}^{-3}$ at 12 km, so it appears that about half of the available condensation sites grew to sizes larger than $0.20 \mu\text{m}$ at this altitude on 9 September. The unusual absence of small particles in the 10–11.5-km region is probably associated with attachment to ice crystals in the $r \geq 5.0\text{-}\mu\text{m}$ layer at this altitude (see Fig. 2). The $1.0\text{-}\mu\text{m}$ profile in Fig. 2 indicates that at 11 km about one half of these particles have radii greater than $5 \mu\text{m}$ whereas above this altitude (11.5–13 km) all of these particles have radii less than $5 \mu\text{m}$ and, as the $r \geq 2.0\text{-}\mu\text{m}$ profile indicates, are essentially all between 1 and $2 \mu\text{m}$. Because there is insufficient sulphuric acid vapour to constitute the mass in the 1–2- μm particles observed at 11.5–13 km, and because it is probably too warm (-78 to -79°C) at this altitude to condense water, these particles may represent the condensation of nitric acid trihydrate (NAT), which can form at temperatures about 7°C higher than the water ice point⁶. However, if sufficient water vapour was mixed up into the 10–11.5-km region to form water ice at -80 to -84°C , as indicated by the $5.0\text{-}\mu\text{m}$ data, it is possible that enough water vapour was present at 11.5–13 km to initiate water condensation at these higher temperatures. The lack of vertical mixing above 11.5 km and the abrupt reduction in particle production at this altitude, as indicated by the minimum in $1.0\text{-}\mu\text{m}$ concentration, makes the latter unlikely. Figure 2 also indicates an $r \geq 1.0\text{-}\mu\text{m}$ layer between 19 and 20 km (another layer was observed between 21 and 22 km) where it would be too dry (frost point $\sim -90^\circ\text{C}$ (ref. 7)) for water ice particles to form at the observed temperature of $\sim -80^\circ\text{C}$. Thus, the most reasonable conclusion is that the enhanced aerosol observed throughout most of the stratosphere on 9 September was composed of NAT.

Figure 3 shows the size distributions for the 11- and 12-km data (0.5-km averages) on 9 September, compared with the 4 September size distribution of the normal sulphate layer at 12 km. The enhanced distributions of 9 September are bimodal. At 11 km, the ice crystals have suppressed the sulphate distribution through attachment. Fitting a log-normal size distribution to the large-particle ($r \geq 1.0 \mu\text{m}$) mode at 11 km gives a mode radius of $5 \mu\text{m}$, a distribution width of 1.5 and a total concentration of 0.03 cm^{-3} , which results in a surface area density of $13 \mu\text{m}^2 \text{ cm}^{-3}$ and a mass mixing ratio, for water composition, of 0.10 p.p.m., which is only a small fraction of the 2–3 p.p.m. of water vapour available⁷.

For the bimodal distribution at 12 km, the small-particle mode can be characterized by a mode radius of $0.19 \mu\text{m}$, a width of 1.28 and a total concentration of 20 cm^{-3} , which results in a mass mixing ratio of 3.3 parts per 10^9 (p.p.b.). The large-particle mode at 12 km has corresponding values of $0.83 \mu\text{m}$, 1.34,

0.3 cm^{-3} and 4.6 p.p.b. Combining the mass in the two modes gives 7.9 p.p.b., which, assuming an NAT composition, implies a value of 2 p.p.b. by volume of HNO_3 . Thus the particles in these clouds have probably incorporated a sizeable fraction of the available HNO_3 vapour. Although there is more mass in the large-particle mode at 12 km, the small-particle mode dominates the surface area density ($3.1 \mu\text{m}^2 \text{ cm}^{-3}$ and $10.2 \mu\text{m}^2 \text{ cm}^{-3}$ respectively for the two modes). This suggests that in a typical cloud undergoing particle growth in the lower stratosphere, area-dependent phenomena such as heterogeneous chemical reactions are controlled by numerous small particles rather than by a few large particles, in contrast to what has been assumed previously⁸. Thus reductions in ozone owing to heterogeneous chemistry in such clouds should correlate best with the small-particle mode.

Figure 4 compares the 10–20-km ozone partial pressure and $r \geq 0.20\text{-}\mu\text{m}$ aerosol profiles. Negatively correlated layers are apparent at 12.7, 14.1, 15, 16.9 and 17.2 km. Correlations are weak or absent for the $1.0\text{-}\mu\text{m}$ aerosol data, as expected from surface-area considerations. To account for this correlation by quasi-horizontal advection, as has been observed in the past⁹, would require a source region unusually rich in particles and poor in ozone. As these observations were made near the centre of the vortex, such ozone depletion cannot be explained by assuming that we are observing low-latitude air; furthermore, such enhanced particle concentrations have never been observed in the stratosphere except after major volcanic eruptions and in

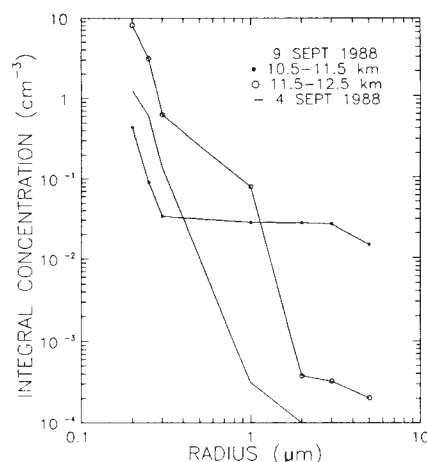


Fig. 3 Integral size distributions for the particle layers observed in polar stratospheric clouds on 9 September and in the undisturbed sulphate layer at 12 km on 4 September.

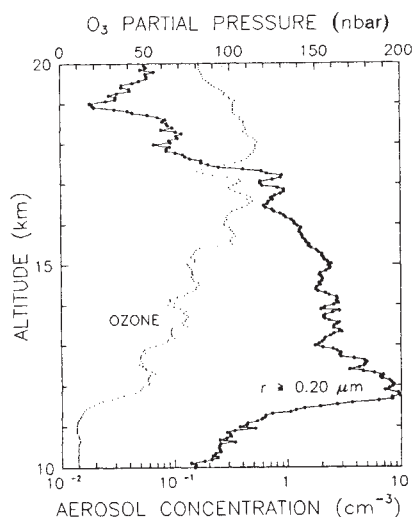


Fig. 4 Ozone partial pressure and $r \geq 0.20\text{-}\mu\text{m}$ aerosol profiles between 10 and 20 km at McMurdo Station on 9 September 1988. Note the inverse correlations at 12.7, 14.1, 15, 16.9 and 17.2 km.

conjunction with very cold regions of the Antarctic polar vortex⁵. No such eruptions were reported at this time, and particle concentrations had returned to normal values when next measured on 27 September.

These measurements thus constitute the first evidence that ozone is reduced directly within polar stratospheric clouds, in contrast to the comparatively loose correlation assumed previously. This explains why ozone depletion takes place in the lower stratosphere, where the abundance of free chlorine may appear to be too low for conventional ozone depletion chemistry to take place. Temperatures low enough for NAT to exist are present in the lower stratosphere well into October, thus prolonging ozone depletion in this region as observed.

The close anticorrelation with aerosol surface area suggests a direct process whereby ozone molecules are destroyed following collision with particles. Such a process, however, could continue through the winter. As depletion does not begin until early September, photochemistry is clearly involved. It is possible that in the lower stratosphere active chlorine exists only within the denser clouds, in which nitric acid has effectively been removed, whereas above 17 km it exists more uniformly. This may be related to the occurrence of apparently more extensive haze- or veil-type polar stratospheric clouds at higher altitudes, compared to the denser, more inhomogeneous clouds in the lower stratosphere^{3,5,7-9}. It would suggest a process that would result in a high degree of spatial inhomogeneity in the ozone distribution of the lower stratosphere and could contribute to the high degree of ozone layering observed in the Antarctic lower vortex^{3,4}. Although most of this structure is believed to be related to ozone emanating from the vortex boundary⁴, layering in the Antarctic lower-stratospheric ozone distribution has been observed to some extent everywhere within the vortex^{3,4,9-11}. This phenomenon may be more readily observable in years when the QBO is in an easterly phase (for example, 1986 and 1988), during which warmer temperatures result in reduced denitrification during the winter.

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Characterization of a new helical smectic liquid crystal

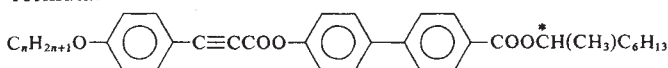
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The discovery of the first liquid-crystalline material in 1888^{1,2} also heralded an age of fascination with chirality and optical activity in ordered fluids. The cholesteric mesophase, which was the first liquid crystal to be found, exhibits form optical activity by virtue of a helical arrangement of its constituent molecules. One hundred years after the discovery of this first liquid crystal, we report the discovery of a new helical smectic liquid crystal, the smectic-A* phase. In this phase the lath-like molecules are arranged in layers with their long axes on average normal to the layer planes. Parallel to the layers there is a helical ordering of the molecules (Fig. 1). We suggest that this phase may be described by a model in which grain boundaries of screw dislocations rotate blocks of layers with respect to each other.

This novel phenomenon was found to occur in an homologous series of ferroelectric liquid crystals, the R- and S-1-methylheptyl 4'-[(4'-n-alkoxyphenyl)propionyloxy]-biphenyl-4-carboxylates (nP1M7). These materials were prepared by the esterification of a variety of 4-n-alkoxyphenylpropionic acids with R- or S-1-methylheptyl 4'-hydroxybiphenyl-4-carboxylate (ref. 3, and M.A.W. and S.M.S., to be published). The helical A* phase was found in the n-tridecyloxy, n-tetradecyloxy and n-pentadecyloxy homologues, which have the general chemical formula:



and exhibit the phase transitions:



where the new helical phase is denoted as A*, the ferroelectric phase as C* (see Fig. 1)⁴, and two further hitherto unclassified helical smectic modifications as S3 and S4 (ref. 5).

Evidence from optical microscopy was obtained for the helical nature of the phase formed first on cooling the isotropic liquid of either the R- or the S- enantiomers of these esters. For example, in a simple experiment in which the n-tetradecyloxy member was sandwiched between clean glass plates, heated to form the isotropic liquid and allowed to cool into the new phase, the phase was found to separate from the liquid in two distinct ways, as shown in Fig. 2a. In this photomicrograph the A* phase exhibits two distinct textures, one a platelet texture and the other a Grandjean plane texture⁶. The platelet texture resembles that of Blue Phase I, whereas the Grandjean plane texture appears iridescent and similar to that of the cholesteric phase. As the plane texture nucleates and grows, striations within the texture are seen. These bands possibly correspond to integral multiples of the pitch of the helix of the phase. Subsequent cooling produces a transition to a chiral smectic-C* phase, which exhibits typical banded, focal-conic and iridescent plane

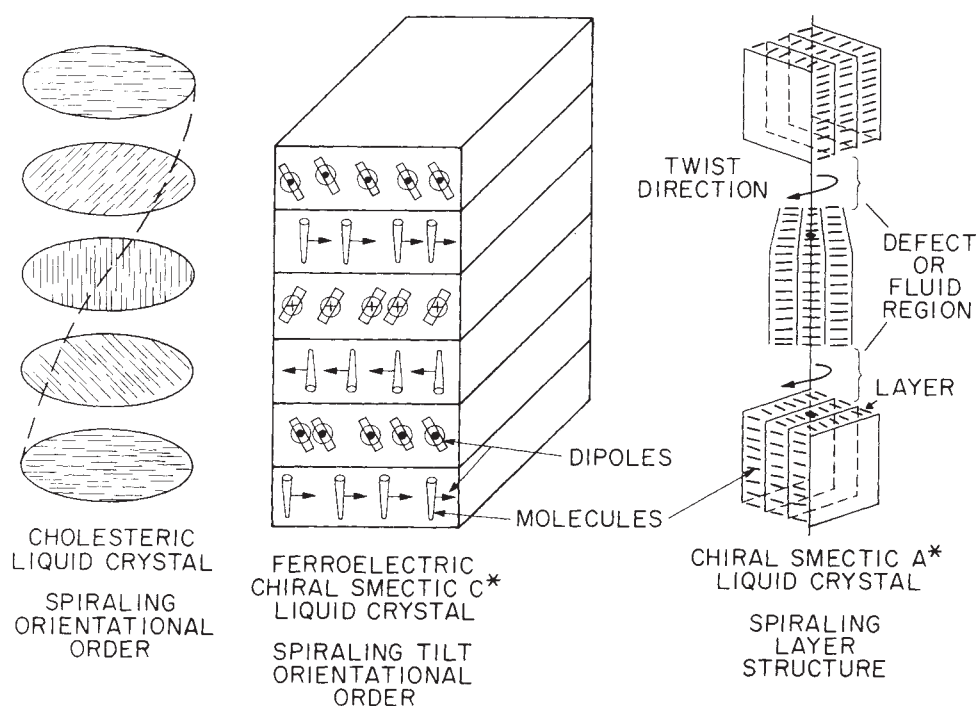


Fig. 1 A pictorial representation of some helical liquid crystal phases, including the cholesteric, the chiral C*, and the chiral A* modifications.

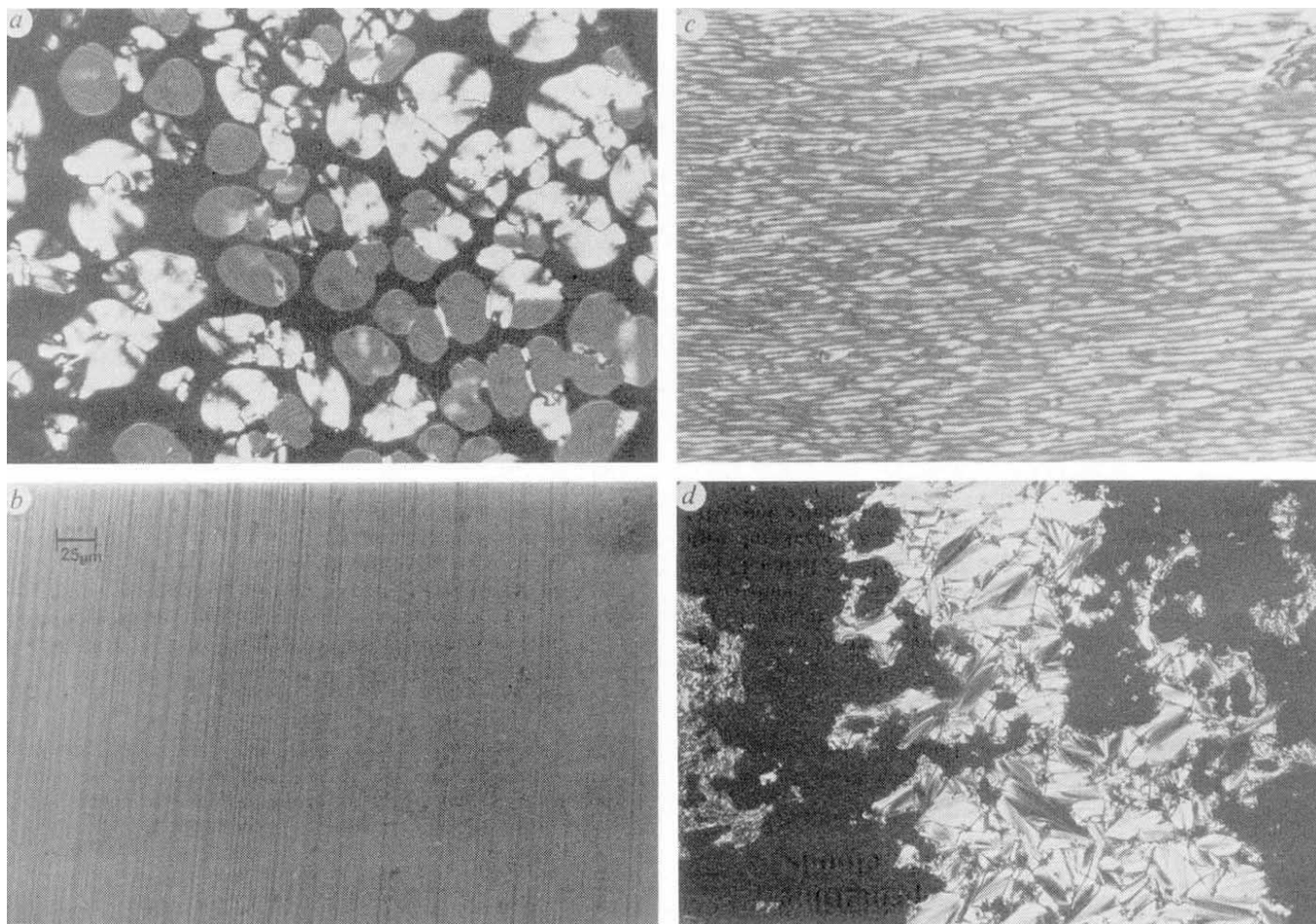


Fig. 2 *a*, The A* phase separating from the isotropic liquid ($\times 100$). *b*, An aligned texture of the C* phase of the *R*-tetradecyloxy homologue. The vertical lines are related to the pitch of the helix of the phase. *c*, The same area as in *b*, but the material is now in the A* phase, and the pitch bands are now orientated horizontally. In both photomicrographs, the material was confined in a cell constructed of conducting glass. The plate separation was 10 μm . A holding voltage of 5 V d.c. was applied to the cell in both cases ($\times 100$). *d*, A contact preparation between the *R*- and *S*-enantiomers of 1-methylheptyl 4'-[(4''-*n*-tetradecyloxyphenyl) propionoyloxy]-biphenyl-4-carboxylate. The centre of the specimen has the texture of the A phase of the racemic modification, whereas the outer regions have the A* texture of the enantiomers ($\times 100$).

textures. The striations on the focal-conic domains, which mirror the positions of the molecular layers, are due to helical pitch bands and dechiralization lines⁷. On cooling through the $A^* \rightarrow C^*$ transition, the focal-conic domains of the C^* phase grow in the regions of the specimen where the Grandjean texture of the A^* phase was present initially. For the Grandjean texture the axis of the helix of the A^* phase is normal to the glass plates, but in the focal-conic domains of the C^* phase it can be seen, from the pitch bands and dechiralization lines, that the helical axis is approximately parallel to the glass. Thus, if the integrity of the layers is maintained through the phase transition, the helical axes in the A^* and C^* phases must be mutually perpendicular.

The position of the helical axes in the two phases was confirmed in a separate experiment, in which the material was aligned in a cell constructed from electrically conducting glass. The internal surfaces of the cell were unidirectionally buffed to create sites for planar, homogeneous growth of the liquid crystal^{8,9}. Epitaxial growth from the liquid resulted in the formation of a Grandjean plane texture. Rotation between crossed polarizers produced no extinction, indicating that the molecules were symmetrically disposed to the viewing direction. This property is typical of a helical structure⁹. As expected, cooling into the smectic- C^* phase produced a focal-conic texture¹⁰. However, even though the molecules were confined by the aligning agent to lie parallel to the plates of the cell, there was a random orientation of the focal-conic domains. This means that molecules did not lie in a preferred direction within the plane of the cell. This texture is similar to that obtained when the C^* phase is formed directly from the cholesteric phase⁹.

Application of an electric field to the specimen in the ferroelectric C^* phase, for conditions close to the $C^* \rightarrow A^*$ transition, produced an aligned sample⁹. In the presence of a small holding d.c. voltage (1 volt per micrometre of cell thickness) dechiralization lines were observed. These lines were oriented at approximately the tilt angle of the C^* phase to the buffing direction, and were parallel to the molecular layers (Fig. 2b). The axis of the helix is therefore in the plane of the cell and perpendicular to the layers. On heating the specimen into the A^* phase, the lines became oriented approximately perpendicular to those in the preceding C^* phase, thereby giving a texture reminiscent of the cholesteric finger-print texture (Fig. 2c). This confirms that the helical axes in the two phases are oriented perpendicularly. Thus, the helix lies in the plane of the layers in the A^* phase. The two relevant textures are shown in Fig. 2b and c, in which are displayed equivalent areas for each phase. When the holding voltage is relaxed, the A^* phase reverts to a plane texture.

For the purposes of phase classification, the new A^* phase can be clearly distinguished from the cholesteric phase in two different experiments. One of these examines the properties of the racemic modifications of these compounds and the other probes the structure of the phase by X-ray diffraction.

The racemic modifications of these materials can be obtained either by synthesis from racemic starting materials or by mixing the R - and S -enantiomers. In the first case the synthetically prepared material was found to exhibit normal A and C phases. In the second, the liquids of the R - and S -enantiomers can be sandwiched between glass plates as before and cooled into their liquid-crystalline phases¹¹ (Fig. 2d). At the junction of the two materials, a racemic modification appears. The proportions of enantiomers in the mixture change with distance from this interface so as to eventually give one or other of the pure enantiomers. Thus a concentration gradient can be observed in the microscope, and the phase changes that occur with varying concentration can be studied as a function of temperature. At the interface, the racemic mixture exhibits the A phase, which was identified from its focal-conic texture. Moving away from the interface there is a continuous change in texture from a homeotropic to a platelet texture as the A^* phase appears (Fig. 2d). As there are no abrupt changes in the texture to indicate

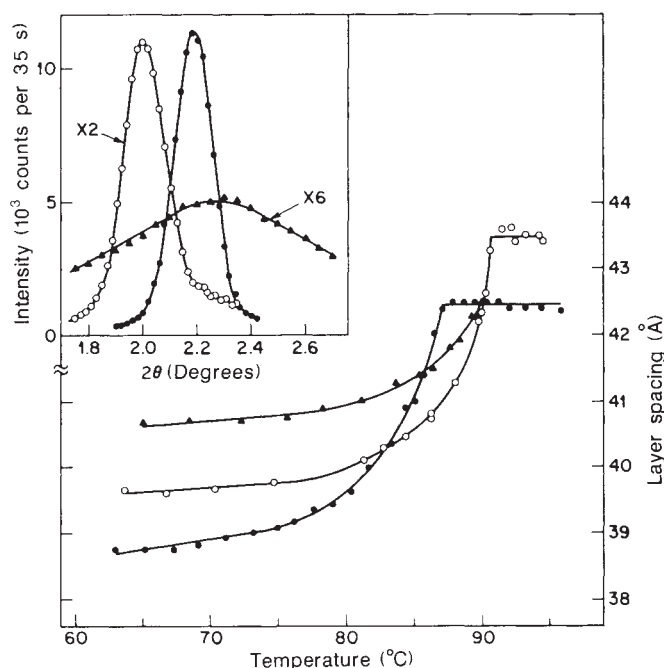


Fig. 3 The temperature dependence of the layer spacing for the $n = 12$ (filled circles), 14 (open circles) and 16 (triangles) R -enantiomers. The inset shows scans through the layer peak for the $n = 14$ enantiomer at temperatures within the C^* -phase (closed circles; $T = 82.8^\circ\text{C}$), the A^* -phase (open circles; $T = 93.3^\circ\text{C}$), and (just into) the isotropic-phase stability fields (triangles; $T = 97.4^\circ\text{C}$).

a phase change, this observation suggests that the A and A^* phases are continuously miscible¹², thereby classifying the helical phase as A. It is also interesting to note that the A phase formed at the interface of the racemic mixture has a higher transition temperature than the surrounding corresponding pure enantiomers. Thus, the A phase of the racemate appears to be more stable than the A^* phase of the enantiomers. Cooling resulted in a transition to the respective C and C^* phases.

Further evidence for distinguishing the A^* phase from a cholesteric phase was obtained from X-ray diffraction using an 18-kW rotating-anode-based spectrometer. For this study, unaligned samples of the R - n -dodecyloxy, R - n -tetradecyloxy and R - n -hexadecyloxy ($n = 12, 14, 16$) homologues were prepared in 1-mm glass capillaries. A vertically bent pyrolytic graphite (002) crystal was used to focus $\text{Cu K}\alpha$ X-rays to a 0.5×2.0 mm spot on the sample. The scattered radiation was then analysed using slits and a flat pyrolytic graphite crystal. This defined an instrumental resolution of $0.01 \text{ \AA}^{-1}$ full width at half maximum in the scan direction, and $0.02 \text{ \AA}^{-1}$ transverse to the scattering plane.

The primary X-ray study consisted of 2θ scans through the smectic layer peak. The temperature dependence of the layer spacing for the three enantiomers is shown in Fig. 3. The layer spacing for each of the enantiomers increases continuously on heating and, for the n -dodecyloxy and n -tetradecyloxy members, saturates in the A^* phase at a relatively constant value. The lamellar spacing was found to be commensurate with the length of a fully extended molecule.

The inset of Fig. 3 shows layer peak scans taken within the C^* , A^* and isotropic phases of the R - n -tetradecyloxy homologue. Within instrumental resolution, the layer peak widths for the C^* and A^* phases are similar, and the integrated intensities in these peaks differ by only a factor of two. This implies that the A^* layer correlations extend a minimum distance of ten layers, thereby establishing smectic layering as a key structural element of this phase. At a temperature just above the $A^* \rightarrow$ isotropic-liquid transition, a diffuse layer peak is seen with a peak position that is comparable to the layer peak position measured well within the temperature range of the C^*

phase. The nature of this observation will be discussed briefly later.

Thus, the qualitative model that emerges for this new phase is one in which the elongated molecules are arranged in loosely ordered layers with their long axes either normal to or only slightly tilted with respect to the layer planes. In the direction of the layer planes there is a twist in the parallel orientation of the molecules. This twist can be effected either by defects, such as screw dislocations, between short sections of the A phase, or by cholesteric liquid-like regions separating larger smectic-A domains, as shown in Fig. 1. The full structural details of this phase are now being studied by high-resolution X-ray diffraction from aligned samples and by freeze-fracture studies. The results obtained from these investigations will be reported soon.

Finally, we note that de Gennes¹³ has predicted that, just as magnetic flux can penetrate Type II superconductors in a lattice of vortices, twist or bend distortions can be incorporated into a layered A structure by the presence of an array of screw or edge dislocations. A model for such an array was proposed recently by Renn and Lubensky¹⁴. With reference to the structure of the A* phase shown in Fig. 1, their model specifies that grain boundaries of screw dislocations are responsible for rotating the individual 'blocks' of the A* layers with respect to each other. They call the resultant phase a twist-grain-boundary (TGB) phase, and predict that it can occur between the cholesteric and the A phase in a manner similar to the occurrence of the Abrikosov flux-lattice phase between normal and superconducting phases¹⁵. Renn and Lubensky established that the requirement for this intermediate phase to occur is that the Ginzburg parameter, which is equal to the ratio of the twist penetration length (λ_T) to the smectic-A correlation length (ξ), must be greater than $1/\sqrt{2}$.

Consequently, the TGB phase is a feasible model for the A* phase, for the following reasons. First, the X-ray scattering pattern predicted for the TGB phase is consistent with our X-ray measurements on the A* phase. Second, the model predicts a helical structure with the helical axis parallel to the smectic layers, which is consistent with our optical experiments. Finally, we observe the A* phase in the vicinity of an A $\rightarrow$ C* transition, where the A phase is stable over a short temperature range. At such a transition, λ_T diverges, resulting in a large value for the Ginzburg parameter, as required by the theory (R. B. Meyer, personal communication). Assuming that the TGB phase is a viable model for the A* phase, we can explore the possible implications of this phenomenon still further. In particular, we can speculate on the nature of the effect observed near to the clearing point in the isotropic liquid. For the superconducting flux lattice it has been proposed¹⁶ and observed¹⁷ that the vortex lattice can disorder into an entangled or disentangled fluid of vortices. We suggest that a similar disordering of the regular array of screw dislocations in the TGB phase may be the mechanism responsible for the occurrence of the effect observed in the isotropic liquid at a temperature just above the transition from the A* phase.

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Disequilibrium of Holocene sediment yield in glaciated British Columbia

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It is generally supposed that specific sediment yield—the quantity of sediment passing a monitored river cross-section per unit area drained upstream of that section—declines as the area drained increases¹⁻³. Part of the sediment mobilized from the land surface is supposed to go back into storage at field edges, and on footslopes and floodplains. In contrast, we show here that data from British Columbian rivers reveal a pattern of increasing specific sediment yield at all spatial scales up to $3 \times 10^4 \text{ km}^2$. This results from the dominance of secondary remobilization of Quaternary sediments along river valleys over primary denudation of the land surface. The result controverts the conventional model which has been derived from studies of small, highly disturbed agricultural catchments. The rivers are still responding to the last glaciation, giving a landscape relaxation time greater than 10 kyr. This holds profound implications for geomorphological theory and for studies of erosion.

Happ and others⁴⁻⁶ have shown that specific sediment yield presently increases downstream in the south-eastern United States, which they explained in terms of remobilization of sediment eroded from uplands during the first two centuries of European agrarian settlement. Schumm⁷ has asserted that such a complex response to episodes of upland erosion is the usual pattern of river-basin development, but apart from a speculation by Meade⁸ that the response in this region may persist for centuries, little has been said about the timescales of adjustment of the fluvial landscape to perturbations in sediment supply, nor has the conventional model been tested critically in natural landscapes.

Our main data resource is the archive of suspended sediment loads obtained by the Water Survey of Canada since 1965. These data (Fig. 1) have been discussed at length elsewhere^{9,10}. Here we emphasize only that they represent contemporary fluvial sediment transfers in British Columbian rivers. Bedload transport, which is not normally measured, accounts for a comparatively small proportion of the total load¹¹. Although solutes may be significant, they do not alter the pattern presented in Fig. 1⁹. The data have been augmented by results from research studies of small drainage basins^{9,12}, which allow us to extend the domain of drainage areas by two orders of magnitude. We have classified basins according to whether (1) they contain contemporary glaciers, (2) they contain large lakes or reservoirs, (3) they have been disturbed by human land use, including mining and contemporaneous clearance of more than 5 per cent of the forest, and (4) they are undisturbed. No basin has a significant proportion (> 5%) of arable land.

The relation of sediment yield to drainage area (Fig. 1) is complex, but a clear trend emerges for basins that are classified as undisturbed. Only three such basins plot outside this trend. Specific sediment yield increases through all areas up to about $3 \times 10^4 \text{ km}^2$. Between 40 km^2 and 10^4 km^2 , specific yield is approximately proportional to (area)^{0.6}, implying a near-linear increase in sediment yield downstream. The local range of the main trend is between about one-half and one order of magnitude in yield: detailed examination of the data¹⁰ indicates that variable Quaternary and bedrock geology is the cause. There is no systematic association, however, between rock type and topography to explain the overall trend, which is replicated within individual major drainage basins.

On the evidence of Fig. 1, primary denudation, indicated by sediment yield in the smallest basins, is more than an order of

Table 1 Comparison of predicted and measured streambank erosion

River	h (m)	M (m yr ⁻¹)	dY/dL^* (Mg m ⁻¹ yr ⁻¹)	A_d (km ²)	Predicted range of $dY/dL^\dagger$	Comments
Liard and Peace River systems‡						
Sikanni Chief	8.3	2.9	13.8	10,530	7.0–38	Sand bed
Fontas	6.5	2.9	9.9	8,110	5.4–30	Sand bed. M assumed
Muskwa	4.0	2.7	4.0	1,510	0.8–4.1	Gravel bed
Prophet	6.0	2.3	7.0	6,765	5.3–29	Gravel bed
Fort Nelson	7.0	4.4	16.5	47,815	32–173	Sand bed. $A_d > \text{limit}$
			(From actual envelopes in Fig. 1:		16–87)	of increasing yields
Beaton	7.0	0.5	1.9	4,070	1.8–10	Fine gravel. M from Nanson ²¹
Upper Fraser River system						
Upper Eagle	3.5	1.3	1.5	150	0.10–0.6	Gravel bed. Disturbed?
Lower Eagle	5.0	0.7	1.6	1,385	0.6–3.3	Sand bed

h = mean bank height; M = mean meander migration rate on bend axis. Data from refs 14, 15.

* Calculated as $1.5(h-2)M/2$; $1.5 \text{ (Mg m}^{-3}\text{)}$ is the assumed bulk density of bank material, $h-2$ adjusts for bed material in the lower bank, and $M/2$ gives the average erosion rate assuming linear variation around the bend.

† From equations in the text; equation for mainstream length is $L = 1.2 A_d^{0.6}$.

‡ Some of these rivers are identical with ones in our study, but the places of measurement are different.

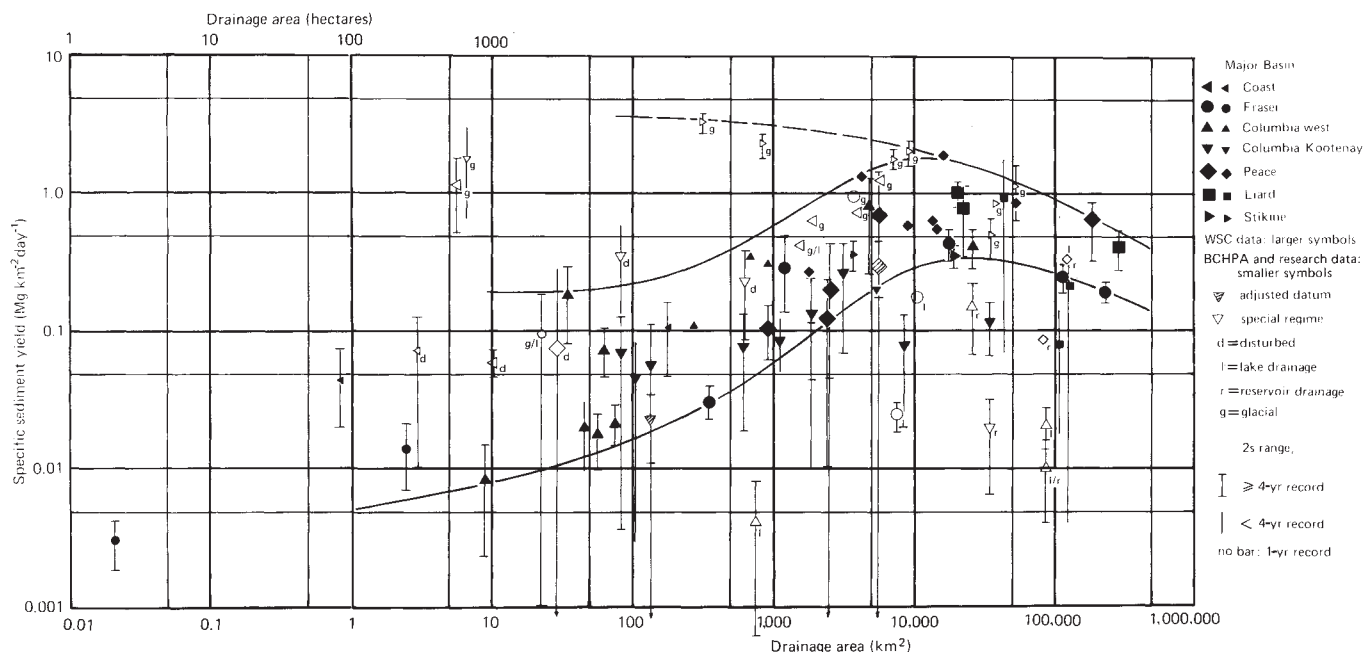


Fig. 1 Specific sediment yield as a function of drainage area for fluvial suspended-sediment-transport records in British Columbian rivers. Closed symbols indicate data for the natural landscape; open symbols indicate yields influenced by natural or human disturbance factors. The main trend of undisturbed yields was sketched by eye. See ref. 10 for a detailed discussion of the data.

magnitude smaller than yield rates in medium to large basins. The latter include a large component arising from secondary remobilization of recent sediments¹³. One must conclude that most of the sediment transported in British Columbian rivers is recruited from the riverbanks and immediate valley-sides. Much of it is derived from the erosion of Quaternary sediments in the valleys and from landsliding of incompetent valley walls.

Thus, the rivers are degrading through valley fill deposits. This is qualitatively evident by the widespread occurrence of Holocene terraces and entrenched trunk streams. We are able to make a quantitative test of our conclusion using data on rates of meander migration obtained by Hickin and Nanson^{14,15}. Assuming a linear relation between specific sediment yield and mainstream length (L) for the rising limb of our trend, total sediment yield Y is proportional to $A_d L$, where A_d is drainage area, so that $dY/dL \propto A_d$. The upper and lower envelopes of our plot are then given approximately by $dY/dL = 36 \times 10^{-4} A_d$

and $dY/dL = 6.5 \times 10^{-4} A_d$, with the coefficients appropriate for units of $\text{Mg m}^{-1} \text{ yr}^{-1}$. dY/dL is the rate of increase in net erosion downstream, or the net rate of bank erosion if the sediment comes from the stream banks. Table 1 shows that the measurements of Hickin and Nanson generally fall within the range predicted by our analysis. All the results demonstrate that riverbank erosion rates are sufficient to support our interpretation of the regional trend of increasing sediment yield.

The conventional model, in which specific sediment yield decreases with basin area, was derived mainly with reference to intensely cultivated regions in which the land surface is highly disturbed and exposed to erosion^{1,2}, so that headwater sediment supply is comparatively unlimited. The sediment yield pattern observed in the natural landscape of British Columbia is more consistent with common notions of pristine headwater streams—particularly in mountains—and sediment-laden trunk rivers. A pattern of sediment yield similar to that observed here has been

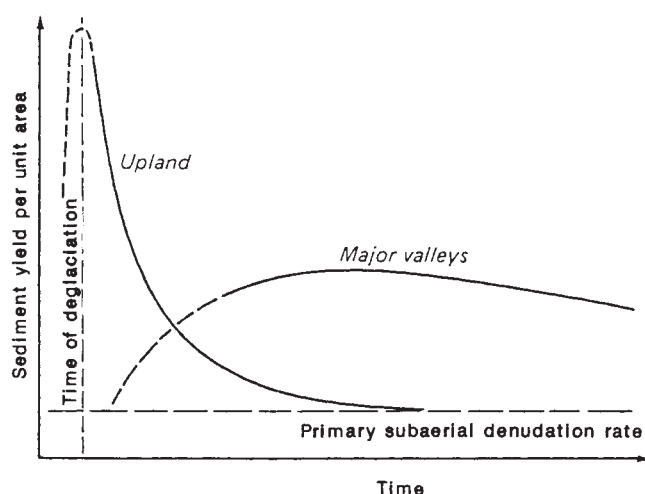


Fig. 2 The paraglacial sedimentation cycle¹⁷, modified to indicate the effect of spatial scale upon the temporal pattern of sediment yield. The time axis spans approximately 10,000 yr.

reported on the Canadian prairies¹⁶, with similar rationalization.

The major rivers are still redistributing sediment that was delivered to the valleys by glacial events more than 10 kyr ago. The transfer and disposition of this material has been termed a 'paraglacial cycle' of sedimentation¹⁷. Originally, evidence of a paraglacial cycle was adduced from outwash deposits and alluvial fans present in the major valleys, and a relaxation time of a few thousand years was inferred from chronostratigraphic markers in the fans. It is now apparent that this timescale is relevant only for the restricted spatial scale of the upland basins that deliver sediment to the fans. An extended paraglacial cycle (Fig. 2) may be said to dominate still in the larger basins. The trend of specific sediment yield in the largest basins ($> 3 \times 10^4 \text{ km}^2$) is consistent with that of contemporary glacierized basins—the most disturbed basins in the region—and conforms approximately with the conventional model. The largest basins are still responding to glaciation. The timescale for ultimate dissipation of this phenomenon appears to be many tens of thousands of years.

It has been supposed for more than 30 years that landscapes adapt so as to reach equilibrium, in some sense, with the imposed environment^{18,19}, which, for stream systems, implies the hydroclimate³. Sediment yield is thereby supposed to be the consequence of erosion from the surface of the land and is supposed to reflect the denudation rate for the prevailing climate and regional geology. This view has been challenged²⁰ on the basis of observations in the south-eastern United States^{4,5}; it certainly is not true at any resolved scale in British Columbia. Fluvial sediment yield at all scales above 1 km^2 remains a consequence of the extraordinary glacial events of the Quaternary Period, rather than of Holocene erosion of the land surface. The results presented here confirm Schumm's contention⁷ that fluvial sedimentation does not reflect contemporary erosion of the land surface at any timescale below that for significant evolution of the entire land mass, which is of the order of several ten thousands of years. The natural landscape of British Columbia is imprisoned in its history.

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Circadian rhythm and light regulate opsin mRNA in rod photoreceptors

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Disk membranes in the outer segment of rod photoreceptors are continuously renewed, being assembled at the outer segment base, displaced outward by new disks and eventually shed at the tip¹. In lower vertebrates, disk assembly occurs with a diurnal rhythm with 2–4% of the outer segment length produced daily^{2,3}. We have discovered that in toad and fish retinas the level of mRNA for opsin, the most abundant protein in rod disks⁴, fluctuates with a daily rhythm and is regulated both by light and by a circadian oscillator⁵. The mRNA level rises before light onset, remains high during the light phase of a diurnal cycle and decreases four to tenfold during the dark phase. In constant darkness, mRNA elevation occurs during subjective daytime. At night, rod opsin mRNA can be elevated by exposure to light.

The level of rod opsin mRNA was measured by hybridization of an opsin antisense RNA probe to blots of total RNA isolated from the retinas of toads (*Bufo marinus*) and fish (*Haplochromis burtoni*). The opsin RNA probe was synthesized *in vitro* using a fragment subcloned from the bovine rod opsin cDNA (Fig. 1). This fragment is highly conserved in both human⁶ and *Drosophila melanogaster*⁷ rod opsin, but badly conserved in the rod opsin homologous proteins: β -adrenergic receptor⁸ and muscarinic acetylcholine receptor⁹. The use of rod opsin probes across species is also justified by the homology between the visual pigment genes in species ranging from archibacteria to mammals¹⁰.

The specificity of our antisense opsin RNA probe, FH97, in toad and fish tissue was verified by hybridization both to blots of total retinal RNA and *in situ*, to retinal sections. At high stringency hybridization (10–12 °C below the calculated transition temperature for the hybrid assuming perfect nucleotide matching¹¹) a single band was detected in autoradiograms of both toad and fish RNA blots (Fig. 1a). In toad RNA blots, a fainter, smaller band was occasionally detected. The labelled RNA was 2.85 kilobase (kb) in the toad and 1.4 kb in the fish, suggesting that in toads, but not fish, the rod opsin mRNA includes an untranslated sequence. *In situ* hybridization at identical stringency revealed that the reactive RNA was located exclusively over the myoid region of the photoreceptor inner segments (Fig. 1b, c).

To determine whether both rod and cone-opsin species were detected by FH97, we used *in situ* hybridization of dark adapted fish retina in which the cone inner segments are displaced relative

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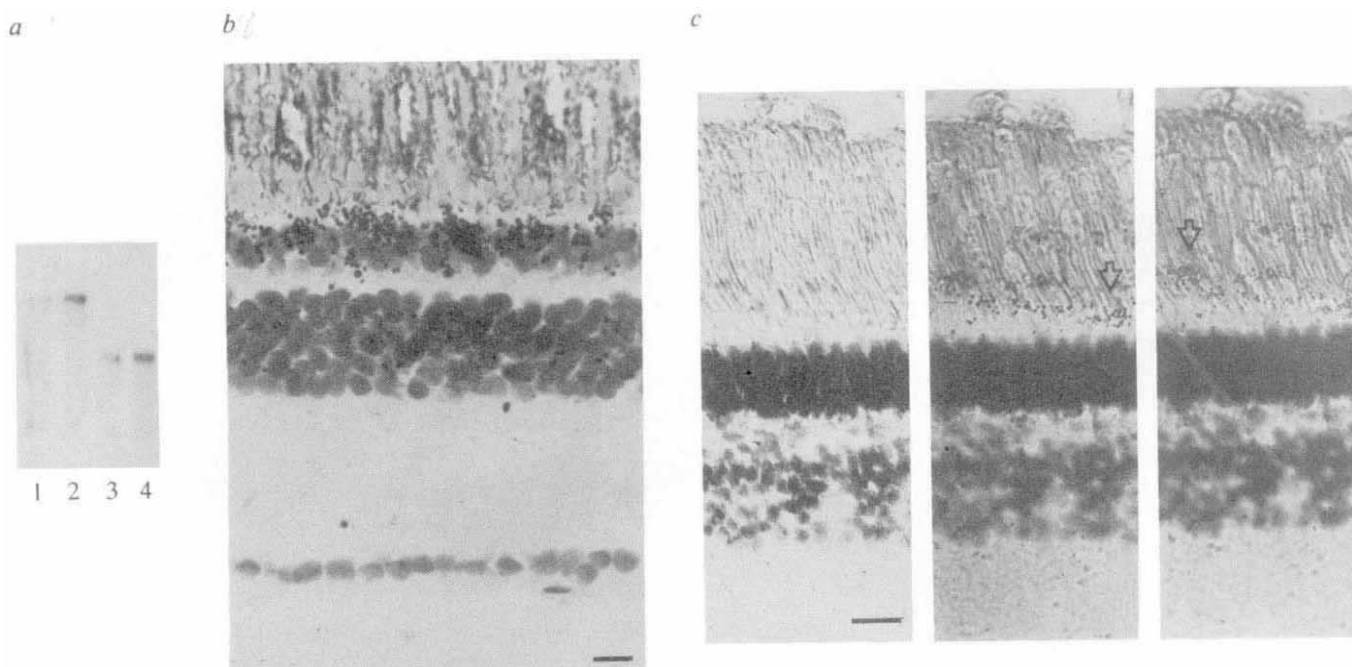


Fig. 1 *a*, Identification of rod opsin mRNA in total retinal RNA blots of toad, *Bufo m.*, and fish, *Haplochromis b.* At high stringency, only a single band was detected in autoradiograms from both toad (lane 1, 4 μ g; lane 2, 8 μ g) and fish (lane 3, 5 μ g, lane 4, 10 μ g). *b, c*, *In situ* hybridization of retinal sections from toad (*b*) and fish (*c*) with 35 S-labelled FH97 antisense mRNA probe. Hybridization was conducted at the same stringency used to probe the RNA blot in *a*. *b*, A light-microscope autoradiograph of a light-adapted toad retina. Autoradiographic grains are exclusively localized over the inner segments of the photoreceptors. *c*, Three photomicrographs of the same field from a dark-adapted fish retina. The left micrograph is focused on the cell bodies, whereas the middle and right micrographs are focused at two different depths within the autoradiographic emulsion. In the middle micrograph, the autoradiographic grains (arrow) are seen distributed over the inner segments of rods. In the right micrograph, the grains (arrow) are localized in discrete groups over the inner segments of cones displaced from the rod inner segments. Scale bar, 10 μ m.

Methods. *a*, Retinas and adhering pigment epithelium were isolated, frozen and maintained in liquid nitrogen until processed. Total RNA was isolated and purified¹⁹, separated by size in a denaturing gel (formaldehyde) and transferred to Nylon 66 membranes²⁰. Blots were prehybridized at 55 °C in 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's, 1% sarcosyl, 5 mM EDTA, 0.1 mg ml⁻¹ yeast tRNA, 0.1 mg ml⁻¹ sonicated salmon sperm DNA. Hybridization was at 55 °C for 20 h in the same solution with 10⁶ c.p.m. ml⁻¹ of 32 P-labelled probe. Final stringency of hybridization was obtained by washing at 75 °C in 0.2 $\times$ SSC with 2.5 mM sodium pyrophosphate, 10 mM EDTA and 0.1% sarcosyl. The opsin antisense mRNA probe (FH97) was synthesized *in vitro* (pGEM4, Promega) from a fragment of bovine rod opsin cDNA obtained by *Fnu*DII-*Hin*II digestion (nucleotides 544-641) of a full cDNA clone¹⁰. This 97 base pair (bp) fragment (FH97), codes for amino acids in the intra-membrane helix V and the loop between helices IV and V on the intra-discal surface of rod opsin²¹. *b, c*, Eyes were fixed by immersion for 50 min in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 followed by 4% paraformaldehyde in the same buffer for 15 min. Tissue was embedded in diethylene glycol distearate (DGD)²². Retinal sections (3 μ m thick) were placed on poly-L-lysine (0.1 mg ml⁻¹) coated slides and DGD removed. Sections were rehydrated, acetylated²³ and prehybridized for 1 h at 45 °C in 60% formamide, 1 $\times$ Denhardt's, 75 mM NaCl, 5 mM Tris pH 8, 2.5 mM sodium pyrophosphate, 5 mM EDTA, 0.1 mg ml⁻¹ yeast tRNA, 0.1 mg ml⁻¹ denatured salmon sperm DNA. The sections were hybridized for 6 h at 45 °C with 35 S-labelled antisense RNA FH97 added to the same medium in the presence of 100 mM dithiothreitol. After hybridization, sections were washed with 500 mM NaCl, 30 mM Tris pH 8 at 45 °C, incubated with RNAase A in this solution at 37 °C for 30 min and washed extensively in 500 mM NaCl, 30 mM Tris pH 8 at 45 °C. Slides were coated with

NTB-2 emulsion (Kodak), exposed at 4 °C, developed and stained with cresyl violet.

to those of the rods¹² and found that the probe labelled both rod and cone inner segments (Fig. 1c). Although FH97 antisense RNA probe could identify both rod and cone mRNA, in blots of total retinal RNA rod opsin mRNA is almost certainly more abundant than cone opsin mRNA. In toads, for example, there are threefold more rods than cones and each rod outer segment is about 30-fold larger than that of a cone. If both cells renew outer segment membranes at the same proportional rate, rod opsin mRNA would be 90-fold more abundant than cone opsin mRNA. For fish, the same reasoning suggests a 20-fold excess of rod over cone opsin mRNA. To test this directly, we hybridized blots to an antisense RNA probe synthesized from either the red or blue human cone opsin cDNA¹³. Under the stringency conditions at which FH97 revealed a single band, neither cone probe yielded any detectable signal, although several bands, including that marked by FH97, were labelled at lower stringencies (data not shown). This failure to detect any bands at high stringency suggests that the amount of cone mRNA present in

our standard retinal RNA blots is below our ability to be detected, as the numerical arguments above suggest.

Rod opsin mRNA abundance during a diurnal cycle was measured in the retinas of animals maintained in 12-hour light-dark cycles and killed every three hours starting half an hour after light onset. In both toads and fish, the mRNA level during the light phase of the cycle was nearly constant and decreased promptly fourfold to tenfold at night (Fig. 2).

Diurnal variation of rod opsin mRNA level could be a response to the light periodicity, or reflect endogenous, circadian signals. Such rhythms are recognized when the time-locked phenomenon occurs periodically in the absence of overt light-dark cycles. We measured opsin mRNA in toads that, after a 12 h:12 h light:dark cycle, were then kept in darkness and killed at three-hour intervals beginning half an hour after the onset of subjective daytime (Fig. 3a) or half an hour after the beginning of the second subjective day (Fig. 3b). The rod opsin mRNA levels in these animals were nearly identical to those of animals

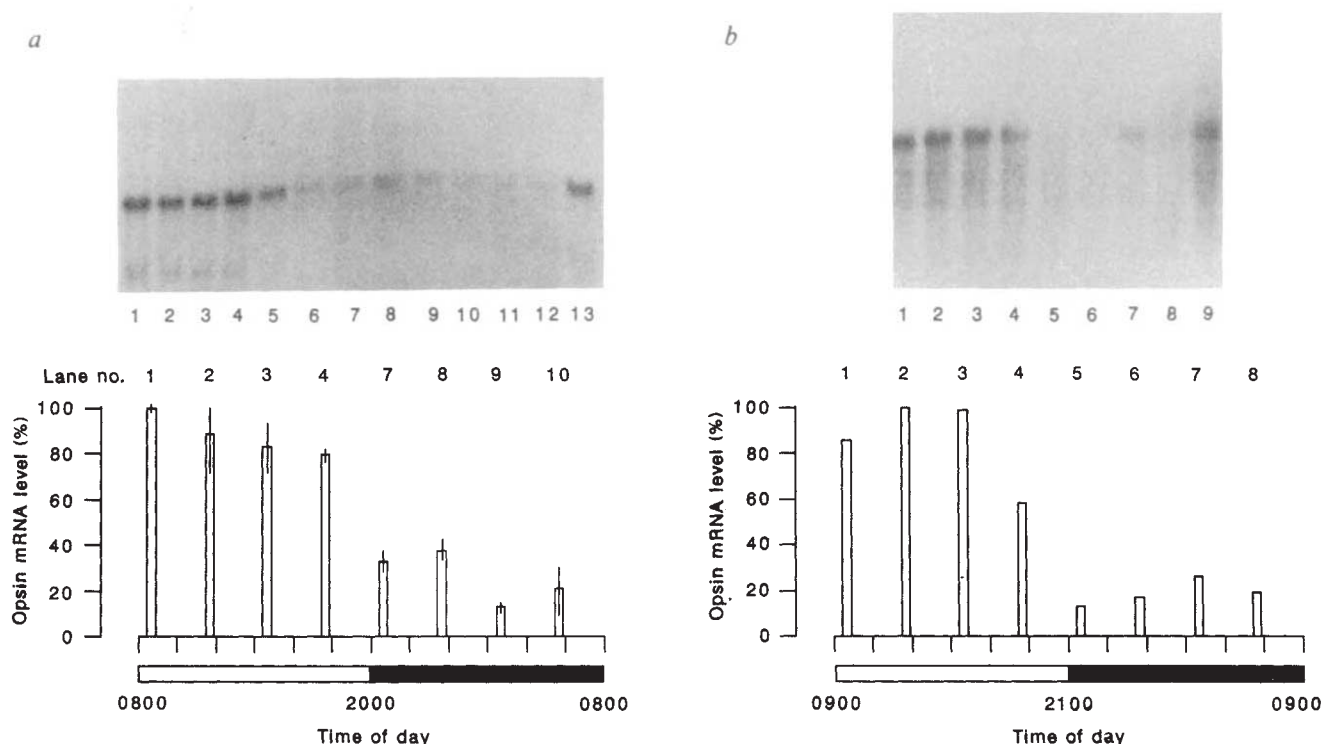


Fig. 2 Diurnal rhythm in the levels of retinal opsin mRNA in toad (a) and fish (b). The blot lanes contained total RNA pooled from two (toad) or six (fish) retinas of animals killed at intervals of three hours throughout a 12 h:12 h light:dark diurnal cycle, beginning 0.5 h after lights on. In the autoradiogram of toad RNA (a) the identity and loading of each lane were: lane 1, 8:30 a.m., 10 μ g; lane 2, 11:30 a.m., 10 μ g; lane 3, 2:30 p.m., 10 μ g; lane 4, 5:30 p.m., 10 μ g; lane 5, 5:30 p.m., 5 μ g; lane 6, 5:30 p.m., 2.5 μ g; lane 7, 8:30 p.m., 10 μ g; lane 8, 11:30 p.m., 10 μ g; lane 9, 11:30 p.m., 5 μ g; lane 10, 11:30 p.m., 2.5 μ g; lane 11, 2:30 a.m., 10 μ g; lane 12, 5:30 a.m., 10 μ g; lane 13, 8:30 a.m., 10 μ g; sampled the following day. In the fish RNA blot (b) all the lanes were loaded with 10 μ g: lane 1 at 9:30 a.m., with lanes 2-9 loaded with samples collected at three hourly intervals after that, so that lane 9 was loaded with a sample collected at 9:30 a.m. the following day. Densitometric analysis of the data and those of repeat experiments, is shown under the autoradiograms. The correspondence between RNA blot lanes and the histogram bars is shown by matching numbers. The density of each lane is expressed as a percentage of the greatest density measured in that autoradiogram. Bar height represents the average of 2-4 experiments and lines indicate the range of values measured. Comparison of RNA samples loaded in various amounts showed a typical error in loading of less than 10% (lanes 4-6).

Methods. All experiments were conducted in the winter, an important point as circadian rhythms and their entrainment can vary seasonally. Animals were adapted to a 12 h:12 h light:dark cycle for at least six weeks. *Haplochromis* were bred from wild-caught stock and maintained at 28 °C. *Bufo* were obtained from commercial sources and maintained at 25 °C in a constant temperature room. Illuminating light was spectrally matched to sunlight and its intensity was 3.1 W m⁻² in the *Bufo* cage and 1.7 W m⁻² at the water surface for *Haplochromis*.

Autoradiograms were analysed with a laser densitometer (Ultrascan XL, LKB222-010, Bromma, Sweden).

that had experienced normal light cycles. Identical results were obtained for fish.

To investigate whether the increase in opsin mRNA during the daytime was synchronized with the light onset we measured the retinal mRNA level in toads killed at 2.5, 1.5 and 0.5 h before, and 0.5 h after light onset. At 2.5 h before lights-on, the opsin mRNA level was low, but it began to increase by 1.5 h before lights-on and was fully elevated 0.5 h before lights-on. Therefore the rise in opsin mRNA level is not activated by light onset but precedes it, suggesting that the levels of opsin mRNA are regulated by an endogenous clock.

We explored the relationship between light and the production of opsin mRNA by exposing toads and fish, entrained to a 12:12 in light:dark cycle, to 0.5 h of light at two different times during their normal dark period: 8 or 3 h before lights-on. In every case, the amount of opsin mRNA in animals exposed to light rose to day time levels (Fig. 3d). Thus, the mRNA level, although controlled by an endogenous rhythm can also respond directly to light.

We are now investigating whether the regulation we have observed in opsin mRNA is shared by other proteins in the rod. In higher vertebrates, diurnal variation in the levels of transducin mRNA^{14,15} and opsin mRNA¹⁵ has been observed, but whether these changes occur with an endogenous rhythm remains unex-

plored. Consistent with the rhythm of opsin mRNA level we describe, the amount of newly synthesized opsin in the frog rod inner segments can change by as much as 13-fold¹⁶. Because similar differences in amount were not observed in the outer segments¹⁶, the variation in the amount of new opsin in the inner segments was presumed to reflect regulation of the size of pools of opsin available for membrane synthesis. Our results suggest however, that changes in opsin content also reflect variation in the levels of its synthesis. Indeed, shortly after light-onset there is fourfold increase in the amount of accumulated opsin in the inner segment which declines only two hours after lights-off¹⁷.

Although the kinetics of each step in outer segment renewal are not known for a single amphibian species, Fig. 4 is a composite representation of the time course of events underlying outer segment renewal from data obtained in *Rana*, *Xenopus* and *Bufo*. Disk membrane assembly¹⁸ is delayed relative to the accumulation of rod opsin in the inner segment¹⁷. Opsin accumulation, in turn, lags behind the initial rise in cytoplasmic mRNA, which precedes light onset.

The selective advantages of diurnal variation in opsin mRNA synthesis and disk membrane assembly are unknown. We speculate that the cone and rod renewal processes are out of phase with one another, rod opsin synthesis occurring mainly during

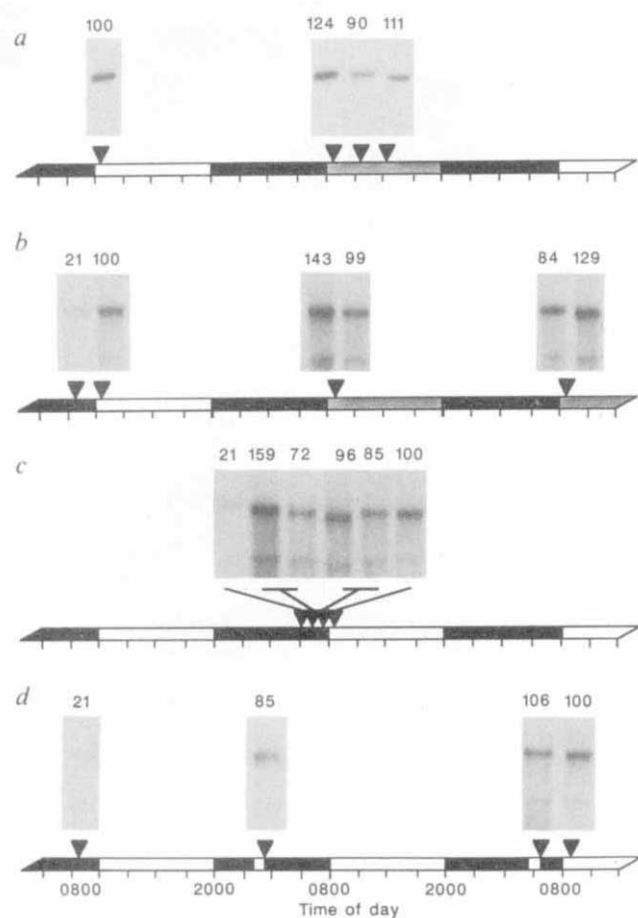


Fig. 3 Opsin mRNA levels are controlled by a circadian oscillator and are responsive to light. In each panel are autoradiograms from the same blot displayed to illustrate the experimental protocol. All lanes were loaded with 10 μ g of toad retinal RNA. Band density relative to that measured 0.5 h after lights-on is expressed as a percentage and shown above each autoradiogram. *a*, The level of opsin mRNA rises in the absence of light during subjective daytime (indicated by the lightly shaded area). From the left, autoradiograms are from samples taken 0.5 h after lights on, and in darkness 0.5 h, 3.5 h and 6.5 h into subjective daytime. *b*, Opsin mRNA level is elevated at the time of subjective light onset after 24 or 48 h of continuous darkness. From the left, autoradiograms are from samples taken: in the dark, 2.5 h before lights turn on; in the light, 0.5 h after lights on; the next two in the dark, 0.5 h into the first subjective day; and the last two in the dark, 0.5 h into the second subjective day. Samples from different animals are shown side by side to illustrate the range and reproducibility of the results. *c*, The increase in opsin mRNA level precedes light onset. From the left, sample identities are: in the dark, 2.5 h before light onset; the next two were in the dark, 1.5 h before light onset; the next two were in the dark, 0.5 h before light onset; the final one was in the light, 0.5 h after the light onset. *d*, Opsin mRNA levels rise in response to illumination during the night. Toads were exposed for 30 min to 2.9 W m⁻² of continuous white light at two different times in the dark phase of their normal diurnal cycle. From the left, sample identities are: in the dark phase of the diurnal cycle (9 h of darkness) in the dark phase of the diurnal cycle (4 h of darkness) after 0.5 h illumination; in the dark phase of the diurnal cycle (9 h of darkness), after 0.5 h of illumination; in the light, 0.5 h after light onset.

the day an cone opsin synthesis at night. If so, animals would have newly synthesized discs in place by dusk each day and rod photoreceptors would not be subject to structural changes at a time when vision is dominated by rod phototransduction.

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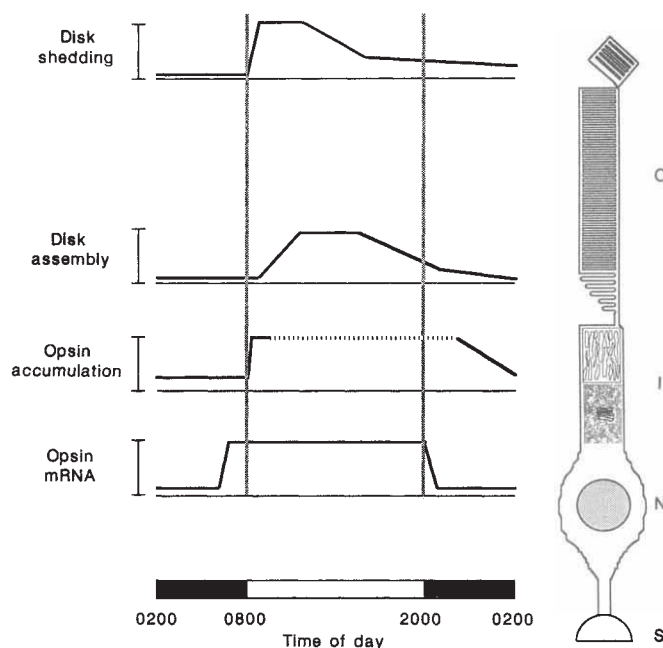


Fig. 4 Schematic illustration of the temporal and spatial relationships of events underlying rod disk assembly during a diurnal cycle based on data from three amphibia genuses maintained in a 12 h:12 h light:dark cycle. A rod is sketched on the right with its major regions indicated: synapse (S), nuclear region (N), inner segment (I) and outer segment (O). The level of opsin mRNA in *Bufo* (compare with Fig. 2 and 3) increases one to two hours before light onset and remains high until shortly after dark onset. Opsin accumulation in the inner segment in *Rana*¹⁷, begins at light onset and continues until two hours after dark onset. The rate of disk assembly in *Xenopus*¹⁸ increases about one hour after light onset and decreases towards the end of the day. Disk shedding in *Xenopus*¹⁸ and *Rana*³ occurs once every four to five days when about 10% of the outer segment length is shed. Shedding begins at light onset and is completed within a few hours.

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A novel eye in 'eyeless' shrimp from hydrothermal vents of the Mid-Atlantic Ridge

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*Rimicaris exoculata*¹ is a shrimp that swarms over high-temperature (350 °C) sulphide chimneys at Mid-Atlantic Ridge hydrothermal fields (3,600 m)¹⁻⁷. This shrimp lacks an externally differentiated eye¹, having instead a pair of large organs within the cephalothorax immediately beneath the dorsal surface of the transparent carapace, connected by large nerve tracts to the supraesophageal ganglion. These organs contain a visual pigment with an absorption spectrum characteristic of rhodopsin. Ultrastructural evidence for degraded rhabdomeral material suggests the presence of photoreceptors. No image-forming optics are associated with the organs. We interpret these organs as being eyes adapted for detection of low-level illumination and suggest that they evolved in response to a source of radiation associated with the environment of hydrothermal vents.

Instead of tubeworms, clams and mussels that are characteristic of vent sites on the East Pacific Rise, the fauna of Atlantic vents is dominated by shrimp. Several species coexist, but *Rimicaris exoculata* is by far the most abundant. As indicated by its specific name, *R. exoculata* lacks eyestalks and corneas¹. Instead, the shrimp possesses a pair of highly reflective patches on the dorsal surface of the cephalothorax. Dissection shows these patches correspond to an anteriorly fused, bilateral pair of specialized organs (Fig. 1), interpreted here as being novel

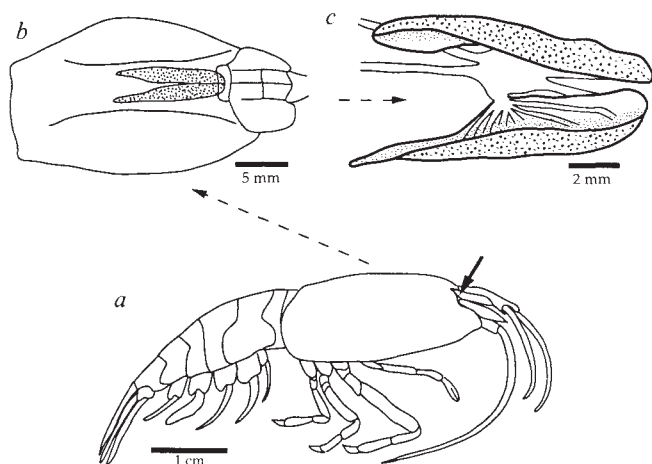


Fig. 1 *Rimicaris exoculata*. **a**, Lateral view. *R. exoculata* lacks eyestalks and conventional compound eyes. Solid arrow points to the location of eyes in the congeneric species *Rimicaris chacei*¹. **b**, Oblique dorsal view showing the location of the novel visual organ (stippled area) underlying the thin transparent carapace. **c**, Dissection of the thoracic eye. The fused anterior tips have been separated along the midline to reveal the underlying connections to the supraesophageal ganglion.

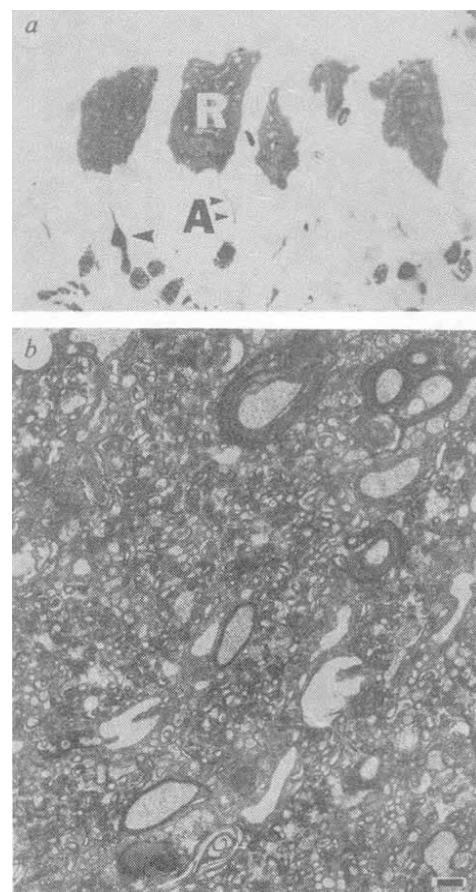


Fig. 2 Structure of photoreceptors of *Rimicaris exoculata*. **a**, Plastic 1-μm section stained with toluidine blue and cut normal to the eye surface. The rhabdomeral segment (R) is filled with a large elaboration of rhabdomere. The arhabdomeral segment (A) is severely attenuated (right arrowheads). The left arrowhead shows a photoreceptor nucleus. No other cellular elements are present. **b**, Electron micrograph of a region of rhabdomeral segment cytoplasm. The vesicles and whorls of membrane probably represent degraded microvilli from the rhabdom. The bar in **b** represents 13 μm in **a** and 0.5 μm in **b**.

eyes. The dorsal quarter of each eye is a compact zone of textured tissue which gives way ventrally to a fibrous web. Fibres of this web coalesce ventromedially into a nerve trunk that enters the fused supraesophageal ganglion in the same position as the optic nerve of other decapods⁸. Examination of 15-μm serial sections of paraffin-embedded tissue stained with haematoxylin and eosin reveals that each fan is composed of ommatidia-like clusters of photoreceptors (5-7 cells per cluster; average, 6). There is no evidence for a lens or dioptric apparatus associated with each 'ommatidium'. Long axes of these 'ommatidia' are oriented perpendicularly to the carapace along the dorsal surface of the organ. Each shrimp has about 9×10^3 receptor cells.

The distal portion of each photoreceptor is a cylindrical rhabdomeral segment filled with microvillar membrane; the proximal portion is a highly attenuated arhabdomeral segment that contains the nucleus (Fig. 2a, left arrowhead). The ultrastructure of the rhabdomeral segment (Fig. 2b) is almost certainly degraded from the living state⁹⁻¹⁶ because the animals were fixed only after exposure to light at the ocean's surface. Nonetheless, the arrays of membranous vesicles and whorls are suggestive of broken-down microvillar rhabdom as found in other invertebrate photoreceptors¹²⁻¹⁶. The reflective property of the organ suggests a tapetal mechanism for increased light absorption by the receptors. As the reflective property is not

preserved in frozen or fixed specimens, we cannot determine the chemical nature of the tapetum, nor the extent to which it contributes to image-formation, if at all. Reflective cells¹⁰⁻¹² and extremely attenuated arhabdomeral segments¹² have been demonstrated in other crustaceans adapted to low ambient light levels. Thus, morphological evidence indicates that the unusual organs in *Rimicaris* are modified compound eyes, specialized for high sensitivity by extreme proliferation of rhabdomeral membrane, deletion of a dioptric apparatus, and presence of a tapetum.

Any organ specialized for photoreception should contain a rhodopsin-like protein. We performed rhodopsin assays on the available light-adapted specimens. Rhabdomeral membranes were partially purified by either isopycnic centrifugation on sucrose gradients¹⁷ or differential centrifugation, and pigment was extracted from the membranes with digitonin. In the presence of hydroxylamine (NH₂OH), light bleaches a shrimp pigment that maximally absorbs at 500 nm and produces a product that maximally absorbs at 366 nm (Fig. 3). The difference spectrum closely resembles that of vertebrate rhodopsins. The location, relative magnitude and shape of the absorption bands indicate that the shrimp pigment is rhodopsin and that the product of the light-induced reaction is all-*trans*-retinaloxime. Formation of all-*trans*-retinaloxime, whose absorption peak is at 365–367 nm (ref. 17), indicates that before irradiation the chromophore is retinal, and is in a *cis*-isomer configuration¹⁷. Formation of retinaloxime also indicates that metarhodopsin is unstable to hydroxylamine under our experimental conditions. In this respect, pigment from *R. exoculata* resembles visual pigments of pelagic euphausiids¹⁸.

Assuming the usual extinction coefficient for rhodopsin¹⁷, 25 pmol pigment was extracted from each organ (50 pmol per shrimp) in the experiment shown in Fig. 3. This is a lower limit, because it ignores the fraction of pigment in the metarhodopsin state and because the limited amount of shrimp tissue prevented us from optimizing experimental conditions. Compared with crayfish¹⁹ and horseshoe crabs²⁰, the eyes of *R. exoculata* contain at least 2–7 times more visual pigment.

Rhodopsin in *R. exoculata* absorbs maximally at a slightly longer wavelength than has been reported for other deep-sea-dwelling crustacea. For example, visual pigments of two species of pelagic euphausiids peak at 485–488 nm in digitonin extracts¹⁸ and in mesopelagic decapods, $\lambda_{\max}$ varies from 485 to 495 nm by microspectrophotometry²¹. Some of these shrimps were also studied with electrophysiological techniques and showed maximal spectral sensitivity at about 500 nm (ref. 22).

Presence of a visual pigment supports morphological evidence that the thoracic organ is an eye; the unusual nature of this eye suggests it has a novel function. In the typical deep sea at depths of 3,600 m, bioluminescence is the only known source of ambient light. Macroorganisms produce flashes of relatively high-intensity light²³ with emission maxima clustered at around 460–490 nm²². In general, the $\lambda_{\max}$ of a visual pigment closely matches the spectral distribution of the ambient light. So it is tempting to relate the visual pigment, with its $\lambda_{\max}$ of 500 nm, to detection of bioluminescent phenomena by the shrimp. But then why should a new sensory organ evolve in place of a normal crustacean eye which is an adequate detector of visible light for most pelagic decapods?

The dominant physical features of the shrimps' environment are plumes of water at 350 °C. Detection of plumes could attract shrimp to feeding areas⁷ and deter them from plunging into water hot enough to cook them. In a medium that does not transmit infrared radiation and in an environment of very steep thermal gradients (>100 °C per cm), detection of plumes with temperature-sensitive receptors is probably inadequate. Can *Rimicaris* 'see' plumes of hot water? Visual detection might be possible if the plumes emit light. Sources of light could be chemical (as in oxidation-reduction reactions or thermoluminescence) or physical (for example, Cherenkov radiation

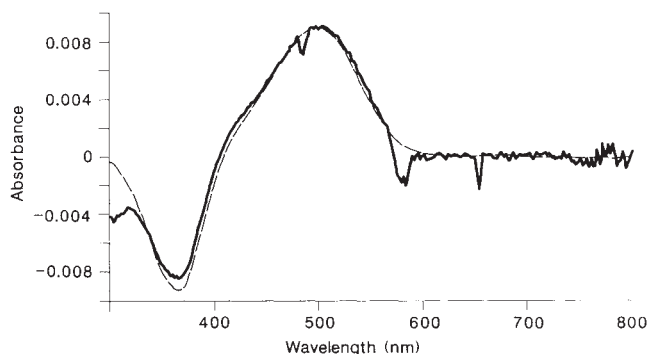


Fig. 3 Bleaching difference spectrum of visual pigment in *Rimicaris exoculata* (solid trace), obtained by subtraction of spectra measured before and after bleaching. Absorption due to reactants is positive and to products is negative. Bleaching destroys a shrimp pigment that maximally absorbs at 500 nm and produces a product that maximally absorbs at 366 nm (Fig. 3). The difference spectrum closely resembles that of vertebrate rhodopsins. The location, relative magnitude and shape of the absorption bands indicate that the shrimp pigment is rhodopsin and that the product of the light-induced reaction is all-*trans*-retinaloxime. The same features are also seen in the difference spectrum of frog rhodopsin (dashed line) normalized to maximum absorbance at 500 nm. The slight mismatch in amplitudes of retinaloxime products may be attributed to a slightly greater (1.08 times) extinction coefficient for shrimp rhodopsin. Spikes at 660, 580 and 490 nm are instrumentation artefacts that regularly appear at these wavelengths.

Methods. All experiments were performed under dim red light with light-adapted animals. Organs of six thawed shrimps were dissected and homogenized in a solution of artificial cytoplasmic medium (80 mM NaCl, 335 mM K₂ethionate, 200 mM sucrose, 4 mM MgCl₂, 10 mM EGTA, 10 mM HEPES titrated to pH 7.4 with NaOH)²⁶ including protease inhibitors (0.5 mM PMSF, 7 μ g ml⁻¹ of pepstatin, 50 μ g ml⁻¹ of leupeptin). The homogenized suspension was centrifuged for 30 min at 10,000g. The resulting pellet was resuspended in a hypotonic medium (10-fold dilution of the artificial cytoplasmic medium) and was centrifuged for 30 min at 51,000g. The final pellet was solubilized for 3 h at room temperature with 2% digitonin in amphibian saline solution (115 mM NaCl, 2.5 mM KCl, 1.5 mM MgSO₄, 10 mM HEPES titrated to pH 7.4 with 4 mM NaOH). Unsolubilized material was removed by centrifugation (2 min at 13,000g). The clear supernatant was used for subsequent analysis on a diode-array spectrophotometer (HP 8452, Hewlett-Packard). After the initial spectrum was recorded, 45 mM NH₂OH was added to the solution in the cuvette to destroy any retinochrome²⁷ present. Subsequently, the cuvette contents were exposed for 5 min to a light source that bleached ~95% of the rhodopsin. For about a 10-min interval both before and after bleaching, the time course of any dark reaction was monitored because the initial tissue homogenate contained relatively large concentrations of water-soluble pigments which could interfere with spectral analysis. In this experiment, the contribution of the dark-reaction was negligible and so no correction was made to the recorded bleaching difference spectrum. We obtained the same bleaching difference spectrum with rhabdoms that were purified by sucrose flotation¹⁷. We specifically searched the rhodopsin extract for the presence of membrane pigments that absorb in the red end of the visible spectrum. Our spectral analysis extended to 900 nm, but no pigment was detected that was preferentially sensitive to the longer wavelengths. The entire protocol from homogenization to extraction was also performed on control tissue derived from the abdomen of the shrimp, where no rhodopsin was detected.

from radioisotopes or thermal black-body radiation). Of these possibilities, only thermal radiation from 350 °C water is a certain source of visible light.

Photons within the visible spectrum represent a very small fraction of the total photon flux emitted by a black body at 350 °C. But they may be sufficient to exceed the presumed threshold in shrimp²⁴. If so, effective photon absorption by *R. exoculata* would be maximal at about 600 nm, given the spectral sensitivity of its rhodopsin. As a test of visibility of hot objects

with a 500 nm pigment, we demonstrated that dark-adapted humans can see a laboratory hot plate heated to 375 °C. *In situ* measurements of ambient light levels and the spectral characteristics and attenuation at hydrothermal vents are only just beginning²⁵.

We have demonstrated that the vent shrimp, *Rimicaris exoculata*, previously thought to be eyeless, has a thoracic eye that is well adapted for detection of very dim light. Thermal radiation from high-temperature plumes at black smoker chimneys could provide the light sensed by the shrimp. The role of the thoracic eye as a visual organ, however, will remain unresolved until more is known about its physiology and its unusual photic environment.

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The visibility of 350 °C black-body radiation by the shrimp *Rimicaris exoculata* and man

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The eye of the 'eyeless' shrimp *Rimicaris exoculata* is unusual in having no image-forming optics and a high concentration of rhodopsin¹. The shrimps swarm around 350 °C hydrothermal 'black smoker' vents in the Mid-Atlantic Ridge². There is no other known source of visible light in the shrimp's environment. The spectral sensitivity of rhodopsin is well matched to typical spectra of bioluminescence of organisms found at lesser depths, but other animals detect such emissions without the unusual features of the *R. exoculata* eye¹. These two features are most easily understood

as an adaptation for the detection of extremely faint sources of light. Physical calculations presented here indicate that the shrimp could see the black-body radiation of the 350 °C vents, even though these sources are practically invisible to the human eye. This would be useful to the shrimp as it feeds on sulphide-loving bacteria very near to the vents³ but must avoid the lethal 350 °C vents themselves.

In the absence of behavioural evidence, no calculation can prove that the shrimp sees anything. Therefore we take the opposite approach, assuming reasonable values for the few unknown parameters, to show that the known physical constraints do not preclude rhodopsin-mediated detection of 350 °C black-body radiation by *R. exoculata*. We begin with the spectra of black-body emission and rhodopsin photosensitivity, and the amount of rhodopsin (50 pmol) in each shrimp eye¹. We assume only that the black smoker is a black-body radiator, that there is no intervening absorption over visible wavelengths by the deep sea water or the carapace of the eye, that a reflective layer at the back of the eye (which is apparent in photographs of live shrimp¹) reflects all light incident upon it, that the dark light (spontaneous isomerizations) in the shrimp eye is comparable to that in other rhodopsin-based eyes, and that the shrimp's nervous system allows it to count (integrate) isomerizations from the entire eye.

A related question is whether this radiation would be visible to man. Partially dark-adapted operators of the Alvin submarine, parked next to the vent, were unable to see anything without artificial illumination (C. L. Van Dover, J. R. Delaney, L. M. Smith, J. R. Cann and D. B. Foster, manuscript in preparation and ref. 4). They did manage, however, to image the hydrothermal vent itself by a long-exposure photograph on a CCD (charge-coupled device) camera.

The spectral photon radiance of blackbody radiation is:

$$N_{e\lambda}(T, \lambda) = 2c\lambda^{-4}(e^{hc/kT\lambda} - 1)^{-1} \text{ sr}^{-1}, \quad (1)$$

where T is temperature, λ wavelength, h Planck's constant, c the speed of light, k Boltzmann's constant, and sr is a steradian⁵. The dashed curve in Fig. 1a shows this spectrum for a 350 °C black body, relative to its maximum at wavelength 5,889 nm (wavenumber $0.17 \mu\text{m}^{-1}$), well outside what is usually considered the visible range. The relative quantum efficiency $\alpha(\lambda)$ of rhodopsin is shown as a dotted curve, peaking at 500 nm (wavenumber $2 \mu\text{m}^{-1}$)⁶. Absorption is proportional to the product of radiation and quantum efficiency, and is shown as the dotted-and-dashed curve in Fig. 1a, relative to its maximum at 588 nm. Note that although the radiation and efficiency curves have very different peaks, at opposite ends of Fig. 1a, their tails overlap and have nearly complementary slopes, resulting in a nontrivial absorption peaking at an intermediate wavelength within the 'visible' range. We have not taken into account the transmission of the intervening water or optics—the carapace over the eye—because we do not have good *in situ* measurements over the relevant wavelengths. Distilled water is, however, transparent over the relevant range (200–1,500 nm) and the transmission outside this range has a negligible effect on the calculated total absorption at 350 °C.

Taking into account the radiation and efficiency, the equivalent number of 500 nm photons $\text{s}^{-1} \text{ m}^{-2} \text{ sr}^{-1}$ is:

$$N_e(T) = \int_0^\infty \alpha(\lambda) N_{e\lambda}(T, \lambda) d\lambda \quad (2)$$

Assuming the eye is directed toward the 350 °C vent, that the vent is a sphere whose radius subtends an angle ω at the eye, and that the light makes two passes through the rhodopsin (before and after reflection at the back of the eye), the number of photons absorbed is:

$$N_a(T) = 2.9 \times 10^{-6} \text{ m}^2 \text{ sr} \sin^2(\omega) N_e(T) \quad (3)$$

This is plotted in Fig. 1b as a function of temperature, assuming that the vent is sufficiently close to fill the shrimp's visual field,

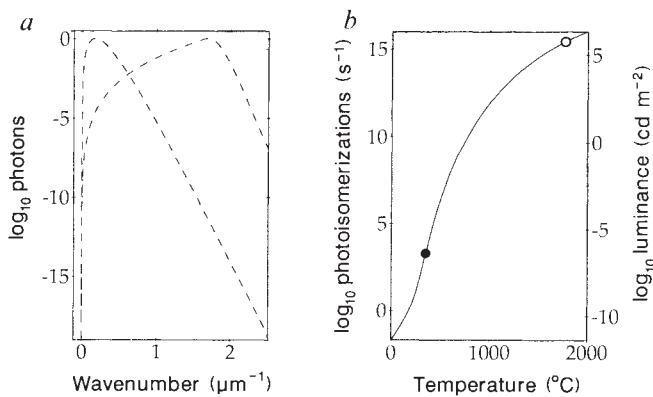


Fig. 1 *a*, The dashed line is the spectral photon radiance $N_{\text{ea}}(T, \lambda)$ of 350°C black-body radiation as a function of wavenumber, $1/\lambda$. The dotted line is the quantum efficiency $\alpha(\lambda)$ of rhodopsin. The dotted-and-dashed line is the product $N_{\text{ea}}(T, \lambda)\alpha(\lambda)$. All three plots are relative to their maximum values. *b*, The number of photoisomerizations per second $N_a(T)$ in the shrimp eye (left-hand scale, assuming $\omega = \pi/2$) and the luminance $L'(T)$ (right-hand scale) as a function of the temperature of the black-body radiator. At 350°C (●) the shrimp eye absorbs 1,800 photons per second under optimal conditions and the luminance is $0.43 \times 10^{-6} \text{ cd m}^{-2}$. The open symbol (○) is explained below.

Methods. Taking into account the angular subtense ω of the target and the area A of the eye, the 500-nm-equivalent photon flux incident on the eye is $N(T) = \pi \sin^2(\omega) \text{ sr} \times AN_e(T)$. The total number of photons that are absorbed and isomerize rhodopsin in two passes (before and after reflection by the reflective layer) will be $N_a(T) = (1 - t_r^2) \gamma N(T)$, where γ is the quantum efficiency for isomerization and t_r is the transmission of the rhodopsin, which is given by $t_r = 10^{-\epsilon C d}$, where ϵ is the molar absorptance at 500 nm, C is the concentration, and d is the path length. As the transmission is nearly 1, we neglect self-screening and make the approximation $(1 - t_r^2) \gamma = (1 - 10^{-2\epsilon C d}) \gamma \approx 2 \ln(10) \epsilon C d \gamma$. The product $\epsilon \gamma$ is called photosensitivity and is approximately $4.06 \times 10^4 \text{ l cm}^{-1} \text{ mol}^{-1}$ at 500 nm for rhodopsin *in vivo*⁹. Thus $N_a(T) = (2 \ln(10) \epsilon \gamma C d) N(T)$, which yields equation 3. The area A and thickness d of the rhodopsin layer in the eye cancel out; all that matters is the total amount of rhodopsin. We estimated the efficiency $\alpha(\lambda)$ of the rhodopsin by a Dartnall nomogram peaking at 500 nm, using Dartnall's long-wavelength extrapolation of that nomogram⁶, that is, an asymptotic gradient $\Delta \log[\alpha(\lambda)] / \Delta(1/\lambda)$ of $1.05 \times 10^{-3} \text{ cm}$. As a check, note that at 2,042 K = 1768.85°C (the open symbol, ○) the luminance is approximately $60,000 \text{ cd m}^{-2}$, in agreement with the 1967 definition of the candela⁵. The scotopic quantum efficiency of a standard human observer $W'_\lambda(\lambda)$ is approximately equal to the 500-nm rhodopsin quantum efficiency curve $\alpha(\lambda)$, except at short wavelengths ($< 500 \text{ nm}$), where lens absorption reduces efficiency, and at very long wavelengths ($> 780 \text{ nm}$) where $W'_\lambda(\lambda)$ is zero⁵. Substituting the standard scotopic relative quantum efficiency function $W'_\lambda(\lambda)$ for $\alpha(\lambda)$ in the definition of $N_{\text{ea}}(T, \lambda)$ would have a negligible effect on the computed luminance at temperatures in the range 500 to 2,000°C, but would have an ever-increasing effect at temperatures below 500°C. At 350°C the scotopic luminance is $0.43 \times 10^{-6} \text{ cd m}^{-2}$ assuming $\alpha(\lambda)$ but would be only $0.16 \times 10^{-6} \text{ cd m}^{-2}$ if we were to assume $W'_\lambda(\lambda)$ instead.

that is $\omega = \pi/2$. At 350°C the photoisomerization rate is $1,800 \text{ s}^{-1}$ (● in Fig. 1b).

We assume that isomerizations caused by photon absorption are indistinguishable from thermal isomerizations. Using the Baylor estimate⁷ of the rate of thermal isomerization of rhodopsin (in monkey rods), 50 pmol of rhodopsin present in the shrimp eye will yield approximately 1,600 thermal isomerizations per second. To detect the 350°C vent, the shrimp would have to discriminate a signal-plus-noise isomerization rate of $1,800 + 1,600 = 3,400 \text{ s}^{-1}$ from a noise-alone rate of $1,600 \text{ s}^{-1}$. A brief integration time of only 20 ms would yield a decision variable

with a signal-to-noise ratio of 6.4 (the mean signal divided by the standard deviation of the noise). This would allow the shrimp to detect correctly the lethal hot water 99.9% of the time, and to mistake ambient for hot water only 2% of the time. The photon flux and signal-to-noise ratio will, however, fall quickly if the shrimp is farther from the vent. Assuming a long integration time of 1 s, then for the shrimp to obtain a minimum useful signal-to-noise ratio of 1 (correctly detect hot water 50% of the time and mistake ambient for hot 16% of the time) the 350°C vent must subtend at least $2\omega = 2.5^\circ$. Thus a 10 cm wide vent could be detected from as far away as 2.3 m.

To assess the visibility of the vent by man we estimate its scotopic luminance:

$$L'(T) = \frac{1,700 \text{ lm}}{W} \frac{hc}{507 \text{ nm}} \frac{N_e(T)}{\alpha(507 \text{ nm})} \quad (4)$$

where lm is a lumen. This is plotted in Fig. 1b over the temperature range 0 to 2,000°C, using the right-hand vertical scale. At 350°C (●) the luminance is $0.43 \times 10^{-6} \text{ cd m}^{-2}$, which is slightly below the average threshold of $0.75 \times 10^{-6} \text{ cd m}^{-2}$ found by Pirenne⁸ for seeing a large long-duration uniform disk by fully dark-adapted observers with natural pupils and unrestricted eye movements. Thus the operators of the Alvin submarine failed to see the vents by naked eye⁴ because the vents were just below the threshold of visibility.

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Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse

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The process of segmentation, in which repeated homologous structures are generated along the anterior-posterior axis of the embryo is a widespread mechanism in animal development. In vertebrates, segmentation is most apparent in the somites and the peripheral nervous system^{1,2}, but the existence of repetitive bulges, termed neuromeres, in the early neural epithelium of vertebrates suggests that the CNS may also be segmented³⁻⁹. Consistent with this, cranial ganglia⁸ and certain neurons^{8,10} are associated with specific hindbrain neuromeres. Here, we report that *Krox-20*, a zinc-finger gene, is expressed in two alternate neuromeres in the mouse early hindbrain. This pattern subsequently decays and *Krox-20* is transiently expressed in specific hindbrain nuclei. In addition, *Krox-20* is expressed in early neural crest cells, and then in the neural crest-derived boundary caps, glial components of the cranial and spinal ganglia. The demonstration that neuromeres are domains of gene expression provides molecular evidence for the segmentation of the CNS.

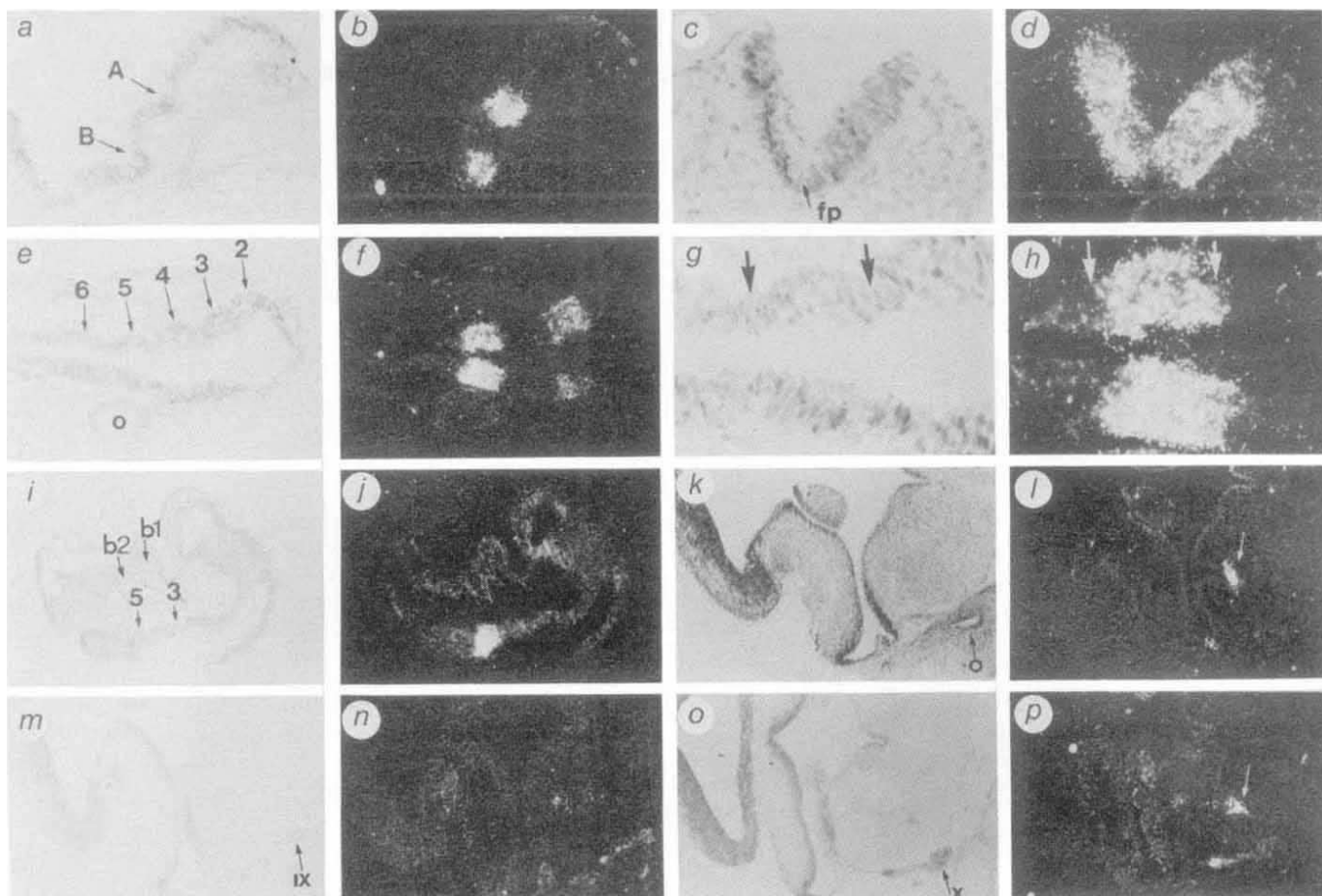


Fig. 1 *In situ* hybridization of ^{35}S -labelled antisense RNA probes for *Krox-20* to sections of mouse embryos. *a, b*, Longitudinal section of 8.5-day-old embryo. *c, d*, Transverse section through proneuromere B of 8.5-day-old embryo. *e, f*, Coronal section through hindbrain of 9.5-day-old embryo. *g, h*, Higher magnification view of neuromere 5 in *e, f*; arrows indicate neuromere boundaries. *i, j*, Parasagittal longitudinal section of 10-day-old embryo. *k-p*, transverse sections through increasing caudal regions of the floor of the hindbrain of a 14.5-day-old embryo. *a, c, e, g, i, k, m* and *o* are brightfield photographs; *b, d, f, h, j, l, n* and *p* are the corresponding darkfield photographs. Embryos are staged according to the developmental ages described by Theiler³¹. Proneuromeres are designated A and B, and neuromeres are numbered 2, 3, 4, 5, 6 (see text for details). fp, floor plate; o, otocyst; b1, 1st branchial arch; b2, 2nd branchial arch; IX, glossopharyngeal ganglion; X, vagal ganglion.

Methods. *In situ* hybridization was performed as described³². The probe corresponded to *Krox-20* 3' untranslated sequences (residues 2,417–2,834 of the cDNA, see ref. 11). Identical expression patterns were observed using a 5' probe (residues 523–883 of the cDNA), although this gave a higher non-specific background (data not shown). No signals above background were observed in sense-strand control hybridizations (data not shown).

Krox-20 was originally identified as a serum-responsive gene in mouse NIH 3T3 fibroblasts, where its transcription is rapidly and transiently activated by the addition of serum or purified growth factors to quiescent cells^{11,12}. *Krox-20* encodes a protein with three zinc fingers similar to those of the transcription factor Sp1¹¹. It therefore seems likely that *Krox-20* encodes a transcription factor that has a role in early events in the resumption of growth in fibroblasts. A 3.2 kilobase (kb) *Krox-20* transcript is detected in the thymus, spleen and testis of adult mice¹¹, and also in developing embryos (data not shown). We used *Krox-20* probes for *in situ* hybridization analysis to examine the spatial and temporal pattern of expression of *Krox-20* during mouse development.

Krox-20 transcripts were not detected in early gastrulation stage (7.5-day-old) mouse embryos (data not shown), but at 8.5 days they were found in two regions of the neuroepithelium of the prospective hindbrain (Fig. 1*a, b*). No other sites of *Krox-20* expression were detected in serial longitudinal and transverse sections of the CNS. The regions of *Krox-20* expression are in the vicinity of two invaginations of the

neuroepithelium termed proneuromeres A and B⁸. Proneuromere B coincides with the centre of a site of *Krox-20* expression, but proneuromere A does not, and analysis of serial sections revealed no simple spatial relationship between proneuromeres and sites of *Krox-20* expression. At this stage, these sites of *Krox-20* expression are 8–10 cells in length. In transverse sections through sites of expression, *Krox-20* transcripts were detected throughout the neuroepithelium, except for the floor plate (Fig. 1*c, d*). By 9.5 days, seven neuromeres had formed in the hindbrain, and longitudinal (data not shown) and coronal sections (Fig. 1*e, f*) revealed that *Krox-20* transcripts are restricted to two alternate neuromeres: neuromeres 3 and 5 of the hindbrain (neuromere 5 flanks the anterior end of the otocyst⁸). The boundaries of *Krox-20* expression coincide with the sulci separating neuromeres (Fig. 1*g, h*). At 10 days of development, *Krox-20* expression has been down-regulated in neuromere 3, but persists in neuromere 5 (Fig. 1*i, j*), and by 10.5 days expression is barely detectable in the hindbrain (data not shown). The down-regulation of expression in neuromere 3 before neuromere 5 may reflect the anterior-posterior direction

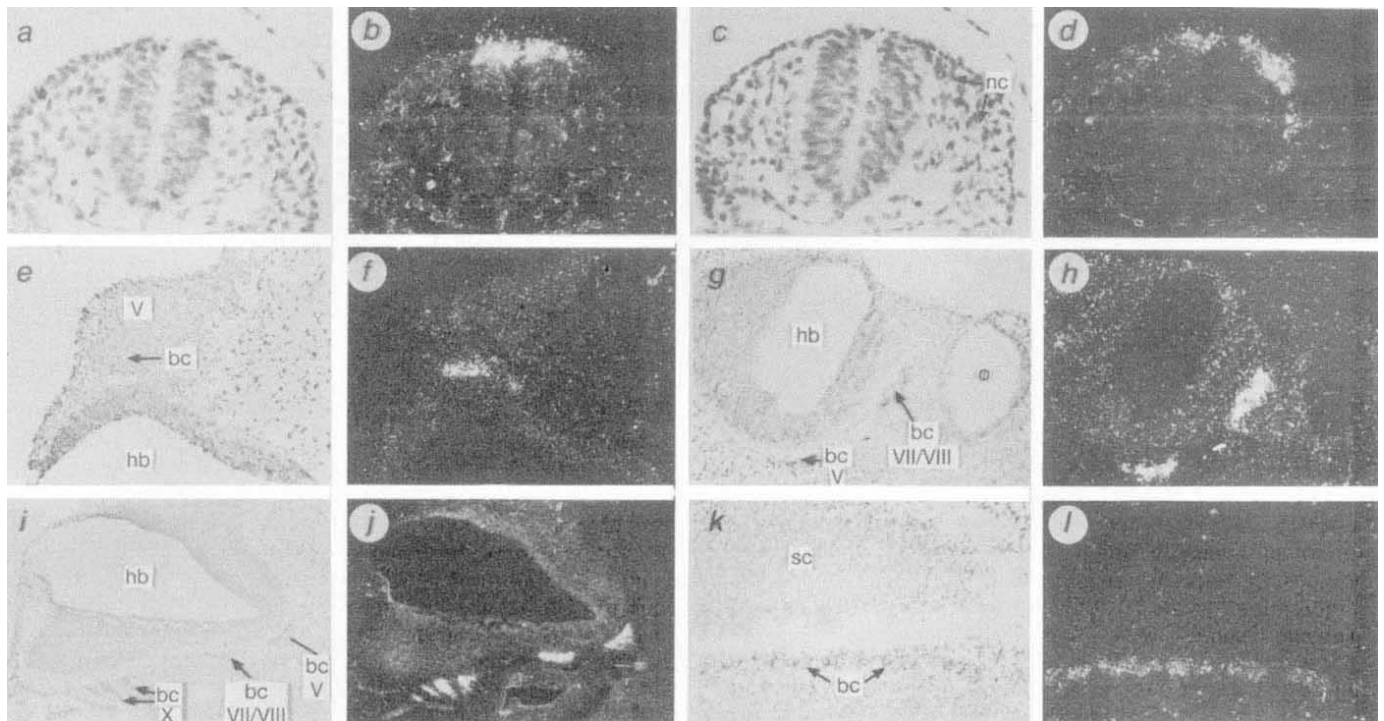


Fig. 2 Expression of *Krox-20* in the developing peripheral nervous system. The pattern of *Krox-20* expression was determined by *in situ* hybridization of radiolabelled probes to some embryo sections. *a, b*, Transverse section of 8.5-day-old embryo through region immediately caudal to proneuromere B (see Fig. 1*c, d*). *c, d*, Transverse section of 8.5-day-old embryo caudal to that shown in *a, b*. *e, f*, Transverse section through trigeminal ganglion of 10.5-day-old embryo. *g, h*, Parasagittal longitudinal section through hindbrain of 10.5-day-old embryo. *i, j*, Parasagittal longitudinal section through hindbrain of 12.5-day-old embryo. *k, l*, Longitudinal section through spinal cord of 14.5-day-old embryo. Arrows indicate sites of *Krox-20* expression. Boundary caps (bc) of trigeminal (V), facial/vestibular (VII/VIII), vagal (X) and dorsal root ganglia are labelled. nc, Neural crest; hb, hindbrain; o, otocyst; sc, spinal cord. Methods were as in Fig. 1 legend.

of CNS maturation. *Krox-20* expression was not detected in the CNS at 12.5 days of development, but at 14.5 days there was expression in two columns of cells in the hindbrain. Serial transverse sections reveal *Krox-20* expression in a dorso-lateral group of cells (Fig. 1*k, l*) that extend from the level of the vestibular ganglion to the glossopharyngeal ganglion. Only low levels of *Krox-20* transcripts are detected immediately caudal to the latter ganglion (Fig. 1*m, n*), but a more ventro-medial column (Fig. 1*o, p*) of *Krox-20*-expressing cells is detected from the level of the vagal ganglion to the caudal end of the hindbrain. The identity of these sites of expression is unclear because of the rapid changes in the formation and migration of hindbrain nuclei at this time. *Krox-20* expression was not detected in the CNS of 16.5-day-old embryos; this was not due to poor retention of RNA as other probes can detect similar levels of transcripts in the hindbrain of 14.5-day-old and 16.5-day-old embryos. Thus, *Krox-20* seems to be transiently expressed in specific nuclei of the hindbrain.

Analysis of serial transverse sections through 8.5-day-old embryos revealed *Krox-20* transcripts in neural crest cells as well. In regions immediately caudal to proneuromere B (see Fig. 1*a-d*), *Krox-20* transcripts are detected in the dorsal neural epithelium (Fig. 2*a, b*), where neural crest cells originate, and, in more caudal sections, early migratory neural crest cells express *Krox-20* (Fig. 2*c, d*). At 10.5 days of development, *Krox-20* expression was detected in boundary cap cells of the trigeminal and vestibular/facial ganglia (Fig. 2*e-h*). Boundary caps are comprised of neural crest-derived glial cells¹³ that are located adjacent to the neuroepithelium at the sites of its penetration by sensory afferent and motor efferent axons¹⁴. By 12.5 days, *Krox-20* expression was detected in the boundary caps of all the cranial ganglia (Fig. 2*i, j* and data not shown) and the

sensory and motor roots of the dorsal root ganglia of the spinal cord (Fig. 2*k, l*, and data not shown). The detection of *Krox-20* transcripts in certain early neural crest cells suggests that these precursors of the boundary cap cells, whose differentiation has been initiated during, or before, neural crest migration. Although much of the neural crest seems uncommitted in its fate at early stages of migration¹⁵, there is evidence for early determination of some neural crest populations^{16,17}. In view of the association of *Krox-20* gene transcription with the G_0 to G_1 transition in fibroblasts^{11,12}, expression of this gene could be associated with the proliferation of boundary cap cells¹⁴. *Krox-20* expression does not, however, correlate with cell division in the neuromeres, and later occurs in post-mitotic neurons.

Despite much debate about the possible developmental significance of neuromeres³⁻⁹, there was no direct evidence for segmentation in the hindbrain of higher vertebrates. Our observation that certain neuromeres are domains of *Krox-20* expression provides molecular evidence that neuromeres are segments. Localized *Krox-20* expression precedes the formation of neuromeres, suggesting that segmentation may occur before it is manifested at the morphological level. The absence of a correlation between *Krox-20* expression and proneuromeres is consistent with the lack of correspondence between these early-forming sulci and neuromeres in the rodent embryo⁹. Recent studies of neuronal development in the chick hindbrain have shown that it occurs in a segmental pattern in hindbrain neuromeres (rhombomeres) 2-6¹⁸. Motor neuron formation is first initiated in neuromeres 2, 4 and 6, and then in neuromeres 3 and 5, yielding an alternating pattern of cellular development. The alternating pattern of *Krox-20* expression in the segmented region of the hindbrain is intriguing, in view of the similar expression pattern of pair-rule segmentation genes in the

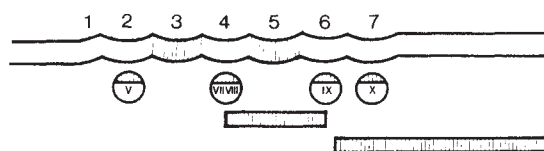


Fig. 3 Summary of sites of *Krox-20* expression, showing the spatial relationship between the various sites of *Krox-20* expression. In the upper part, the neuromeres (1-7) are shown; segmentation at the level of cellular development occurs within neuromeres 2-6 (ref. 18), and *Krox-20* expression occurs in alternate neuromeres within this domain. Below are aligned representations of the cranial ganglia and boundary caps (V-X). Cranial ganglia caudal to the neuromeres are not indicated. The lower part of the figure shows the relative position of the nuclei that express *Krox-20* at 14.5 days of development. The sites of *Krox-20* expression are indicated by shading.

Drosophila melanogaster embryo^{19,20}. It is possible, however, that *Krox-20* expression may be associated with phenotypic characteristics unique to neuromeres 3 and 5. For example, motor neuron formation is delayed in these neuromeres, and they are the only definitive neuromeres not adjacent to cranial ganglia (Fig. 3). There is no obvious relationship between the transient expression of *Krox-20* in neuromeres and, later on, in specific hindbrain nuclei; the rostral column of cells expressing *Krox-20* overlaps with the hindbrain region derived from neuromere 5, but the more caudal nucleus has no such overlap (Fig. 3). Thus, it seems likely that *Krox-20* acts in distinctive cell populations at different stages of hindbrain development. There are other examples of this behaviour for genes with early segment-restricted expression, as many segmentation and homoeotic genes in *Drosophila* are subsequently expressed in certain neurons in all segments²¹.

Our data suggest that *Krox-20*, as a putative transcription factor, could be part of a regulatory network coupled to hindbrain segmentation. The expression of mouse homoeobox (*Hox*) genes in restricted anterior-posterior domains of the neural tube suggests that they may specify position along the body axis^{22,23}. Many of these genes have anterior limits of expression in the hindbrain, although a possible relationship with neuromeres has been observed only for *Hox-1.5*²⁴. It is therefore pertinent to investigate whether *Krox-20* interacts with *Hox* genes or with other members of the mouse zinc-finger gene family²⁵⁻²⁶. One such gene, *mKr2*, is expressed throughout the developing CNS and peripheral nervous system²⁷, and the mouse genome contains at least one other zinc-finger gene closely related to *Krox-20*²⁸⁻³⁰. It will be interesting to determine whether other members of this group are expressed in the developing nervous system and, in particular, whether any have neuromere-restricted domains of expression.

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Transgenic mice overexpressing the mouse homoeobox-containing gene *Hox-1.4* exhibit abnormal gut development

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The mouse homoeobox-containing genes exhibit temporally and spatially specific patterns of expression in embryonic and adult tissues and are thought to be important in regulation of development and cellular differentiation, perhaps by mechanisms analogous to homoeotic genes in *Drosophila melanogaster*¹⁻⁴. There has been no direct demonstration that expression of these mammalian genes can affect developmental processes, however. *Hox-1.4*, like other mouse homoeobox-containing genes, has been shown to be expressed in specific regions of the mid-gestation embryo⁵⁻⁸, but is unique in that its highest level of expression in the adult animal is restricted to developing male germ cells^{5,8,9}. We have introduced a construct carrying the mouse *Hox-1.4* gene into the germ line of mice to begin to identify the cis-acting elements required for proper expression and to assess the consequences of increasing *Hox-1.4* gene expression. The construct was designed to produce normal *Hox-1.4* protein from transcripts that are distinguishable from the products of the endogenous gene. The integrated transgene seemed to exhibit the appropriate tissue specificity of expression, but transcript levels were elevated in certain tissues, particularly the embryonic gut. This overexpression correlated with changes in the normal developmental program of the gut, resulting in an inherited abnormal phenotype known as megacolon.

Hox-1.4 is a member of a cluster of at least six homoeobox-containing genes occupying about 100 kilobases (kb) of contiguous DNA on mouse chromosome 6 (refs 8, 10, 11). Although the putative translation stop codon and other structural features of the 3' end of the *Hox-1.4* gene have been determined, the 5' end has not been characterized. We therefore generated a construct (Fig. 1a) which contained ~10 kb of sequence upstream of the homoeodomain and abuts the upstream *Hox-1.3* gene^{10,12,13}.

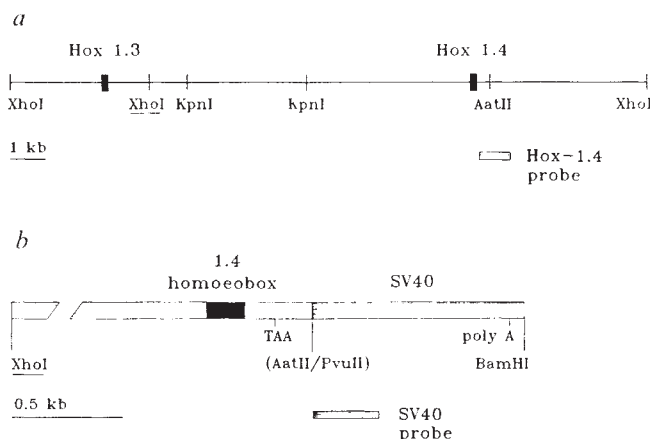


Fig. 1 Map of *Hox-1.4* and construct used to generate transgenic mice. *a*, Map of the *Hox-1.4* gene and flanking regions. Pertinent restriction sites are indicated. *b*, The *XhoI*-*BamHI* fragment that was microinjected to generate transgenic mice.

Methods. A plasmid spanning *Hox-1.3* and *Hox-1.4* was cut at the unique *AatII* site to remove the endogenous poly(A) site of *Hox-1.4* (ref. 20). A *PvuII*-*BamHI* fragment of SV40 (position 3,506 to 2,533) containing a polyadenylation signal²¹ was ligated to the *AatII* site, downstream of the translation stop codon of *Hox-1.4* (refs 9,20). The 'Hox-1.4 probe' in *a* was used to detect endogenous mRNA^{5,20}. To detect transgenic mRNA only, the SV40 probe (Fig. 1b) was used. Both fragments were subcloned into pGEM vectors purchased from Promega.

We assumed that this DNA fragment contains the complete coding sequence of *Hox-1.4* and would be capable of producing functional *Hox-1.4* protein if expressed. To distinguish the transcripts generated by this construct from those of the endogenous *Hox-1.4* gene, about 400 base pairs (bp) of 3' untranslated region of *Hox-1.4* was substituted with simian virus 40 (SV40) T-antigen coding region and poly(A) addition site (Fig. 1b). Transgenic mRNAs should be ~400 nucleotides (nt) longer than endogenous *Hox-1.4* transcripts and easily distinguishable because of the SV40 sequences.

Initially, we characterized two transgenic lines (1974-3 and 1975-2), each carrying multiple copies of the 10 kb *Hox-1.4*-SV construct at a single integration site. Inheritance of the transgenes in the 1974-3 lineage is exclusively by males and is therefore genetically linked to the Y chromosome, whereas the pattern of inheritance in the 1975-2 lineage is autosomal.

The tissue specificity and level of expression of the *Hox-1.4* transgene in adult tissues from both lines was examined by northern blot hybridization analysis. SV40-containing mRNAs were readily detected in testes of the 1975-2 lineage, but not in the other adult tissues examined (Fig. 2a). Occasionally, a low level of SV40-containing mRNA was detected in lung from these transgenic mice, consistent with the low level of expression of the endogenous *Hox-1.4* gene observed in this tissue (R. Krumlauf, personal communication). Recent preliminary data on the pattern of expression of *Hox-1.4*-SV constructs with 5 kb (rather than 10 kb) of DNA 5' to the *Hox-1.4* homoeobox reveal this same pattern of tissue distribution of transgenic mRNAs in the adult animals—highest levels in the testis and barely detectable levels in lung and kidney (another somatic tissue exhibiting low levels of endogenous *Hox-1.4* expression: R. Krumlauf, personal communication; C. M. Viviano and D. J. Wolgemuth, unpublished observations) (data not shown). These observations suggest that the *cis*-acting regulatory elements responsible for the appropriate tissue-specific expression of *Hox-1.4* are present in these constructs. The levels of *Hox-1.4*-SV transgenic transcripts however, in the testes of adult males of the 1975-2 lineage (Fig. 2b), as well as in testes of two additional lines carrying the 10 kb

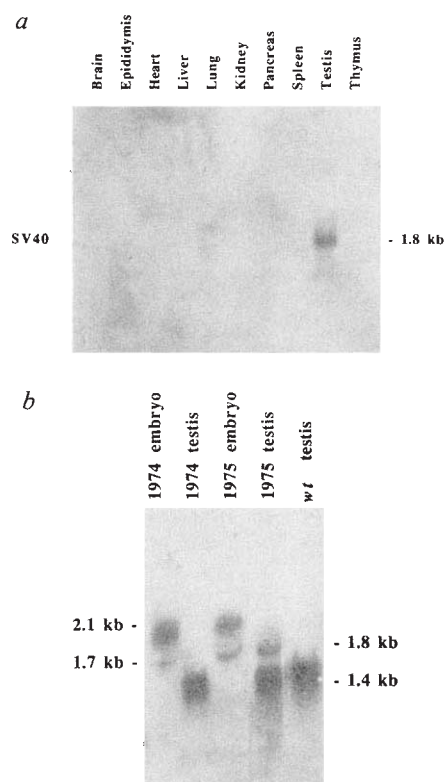


Fig. 2 Northern blot hybridization analysis of expression of the 10 kb *Hox-1.4*-SV transgene. *a*, Expression of the 10 kb *Hox-1.4*-SV transgene in adult tissues of the 1975-2 lineage. Total RNA (25 µg) from tissues of an adult (>45-day-old) male mouse from the 1975-2 lineage was examined for the presence of transgenic mRNA using the SV40 probe. Transgenic mRNAs of the predicted ~1.8 kb size were readily detected in testis but not reproducibly in other tissues. *b*, Expression of transgenic and endogenous *Hox-1.4* transcripts in mid-gestation embryos and adult testes of the 1975-2 and 1974-3 lineages. RNA was isolated from transgenic day-12.5 (day 0.5 is day of plug) whole embryos or from testes of adult animals. Total RNA (~25 µg) was loaded per lane. We used the *Hox-1.4* probe, which detects both transgenic and endogenous *Hox-1.4* transcripts (see Fig. 1). The larger transcripts were transgenic, as confirmed by hybridization with the SV40 probe (data not shown). Transcripts of 1.7 and 1.4 kb were produced from the endogenous gene in day-12.5 embryos and adult testes, respectively, as expected⁵. Transgenic mRNAs of ~2.1 and 1.8 were detected in embryo and testis RNA, respectively, from the 1975-2 lineage, whereas the 1974-3 lineage expressed the transgene only in embryos, not in testis.

Methods. Transgenic mice were generated by microinjection of the 10 kb *Hox-1.4*-SV construct into the pronuclei of C57B1/6 × SJL F₂ fertilized eggs by standard procedures²². Weanlings were identified as transgenic by dot hybridization of DNA collected from tails²². Embryos were identified by analysis of DNA from placenta and extra-embryonic membranes. Tissues were obtained from progeny of founder animals, unless otherwise stated. RNA was isolated by the LiCl precipitation method²³. Poly(A)⁺ RNA was isolated as described²⁴. Total or poly(A)⁺ RNA was electrophoresed on 0.8% agarose/2.2 M formaldehyde gels²⁵, transferred to Genescreen Plus (New England Nuclear), cross-linked under UV light for 7 min, and hybridized at high stringency²⁵ as previously described²⁶. Isolated inserts were labelled by nick translation with ³²P[dXTP]²⁷. Final washes were at 0.1 × SSC, 68 °C. Filters were exposed to XAR film with intensifying screens.

Hox-1.4-SV transgene and two lines carrying the 5 kb *Hox-1.4*-SV transgene (data not shown), were lower than those produced by the endogenous gene. This suggests that elements involved in quantitative regulation of *Hox-1.4* mRNA levels may be missing.

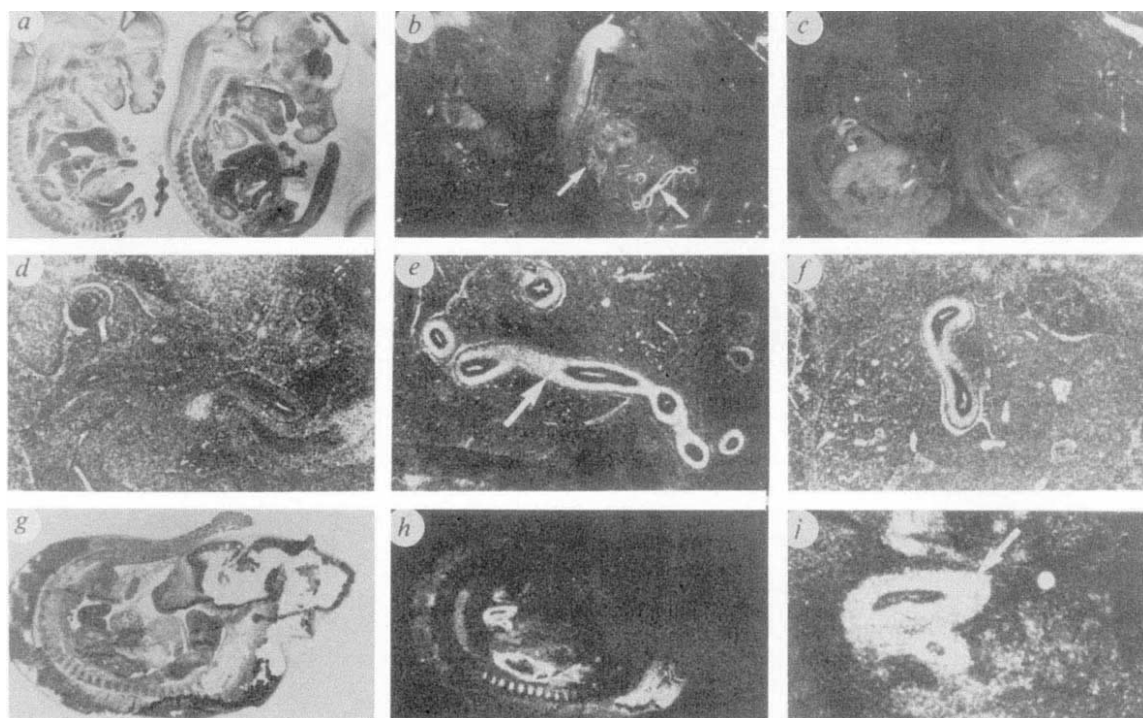


Fig. 3 *In situ* hybridization analyses of endogenous and transgenic *Hox-1.4* transcripts in mid-gestation embryos. *a, b*, Parasagittal sections through day 12.5 embryos of the 1974-3 lineage photographed with bright-field (*a*) or dark-field (*b*) optics. The embryo on the left is a non-transgenic litter-mate of the transgenic embryo on the right. The antisense *Hox-1.4* probe used detects both endogenous and transgenic *Hox-1.4* mRNAs. Localization is clearly seen in the more rostral region of the spinal cord and in the caudal myelencephalon of the wild-type and transgenic embryos. The labelling is more intense in the transgenic mouse, indicating higher levels of *Hox-1.4* mRNA. Elevated levels of expression are also evident in the lung and intestine of the transgenic embryo (arrows). The labelling over the heart is non-specific, as heart is also labelled using the sense (control) probe (*c*). The hybridization pattern of antisense *Hox-1.4* (*d, e*) or SV40 (*f*) RNA in regions of the developing gut of wild-type (*d*) and transgenic (*e, f*) embryos are shown at higher magnification in *d, e*, and *f*. Labelling is restricted to the mesenchymal layer of the developing gut (arrows). In the transgenic embryo, the SV40-containing transgenic mRNAs are readily detected and exhibit localization to the mesenchymal layer (*f*). Expression of transgenic *Hox-1.4* mRNA is also elevated in gut of mid-gestation (day 11.5) embryos of the 1975-2 lineage (*g-i*). *g*, A bright-field photograph of the embryo in *h*; *i*, an enlargement of portions of the gut. The labelling is again restricted to the mesenchymal layer (arrows in *i*).

Method. Procedures followed previous protocols^{6,28}, except where noted. Embryos were recovered at day 11.5 and 12.5 of gestation, fixed overnight at 4°C in 4% paraformaldehyde: 0.1 M phosphate buffer, pH 7.5, followed by equilibration in 30% sucrose in 0.1 M phosphate buffer, pH 7.5. Specimens were embedded in O.C.T. compound (Miles) and sectioned on a cryostat. Sections were collected onto poly-L-lysine coated slides. Hybridization conditions were as described^{9,28}, except that 10 mM dithiothreitol was included in the first post-hybridization wash. RNA probes were synthesized from the T7 or SP6 promoters in the GEM3 plasmid vector to generate antisense or sense RNA using ³⁵S[UTP]. Specimens in panels (*b, d, e*) were hybridized with antisense *Hox-1.4* probe panel (*c*) with sense *Hox-1.4* RNAs; panels (*f, h, i*) with antisense SV40 RNA probe.

There was no detectable expression of the transgene in testes (Fig. 2*b*) or other adult tissues tested (data not shown) of the Y-linked 1974-3 lineage. The lack of expression of the transgene in the Y-linked lineage may be due to the condensation during meiotic prophase of the X and Y chromosomes into the sex vesicle, which is purported to be transcriptionally inactive^{14,15}.

The northern blot analysis further revealed that in mid-gestation embryos of both the 1974-3 and 1975-2 lineages, the levels of transgenic mRNAs were at least fivefold higher than the levels of the endogenous *Hox-1.4* transcripts (Fig. 2*b*). To determine whether the elevated levels of the transgenic mRNAs in the embryos could be attributed to the presence of higher levels of transcripts in tissues normally expressing *Hox-1.4*, to expression of the transgenic *Hox-1.4* in ectopic sites, or to a combination of both, mid-gestation embryos of the 1974-3 and 1975-2 lineages were examined by *in situ* hybridization analysis.

The endogenous *Hox-1.4* gene is expressed at high levels in the developing nervous system of the mid-gestation mouse embryo, especially within the spinal cord, as shown by both northern blot⁵ and *in situ* hybridization analysis^{6,7}. Both the non-transgenic and transgenic 12.5-day-old embryos shown in Fig. 3*b* exhibit the expected abundant expression of *Hox-1.4* in

portions of the spinal cord and myelencephalon (Fig. 3*a, b*). Hybridization signals were also observed in heart. As the signal detected in heart was also observed in experiments using sense *Hox-1.4* probes, however (Fig. 3*c*), it presumably represents non-specific, artefactual hybridization.

In the transgenic embryo, the spatial distribution of *Hox-1.4*-containing mRNAs in the myelencephalon and spinal cord was similar to controls, but the levels seemed elevated (Fig. 3*b*). *Hox-1.4*-containing mRNAs were more readily detected in the lung of the transgenic rather than non-transgenic embryo, suggesting that there are slightly elevated levels of expression in this tissue as well (Fig. 3*b*). We believe that the levels of *Hox-1.4* mRNA are elevated at least twofold in both the central nervous system and lung of the transgenic embryos because the grain density was consistently higher and the time required to obtain a signal was consistently shorter than with litter-mate controls using the *Hox-1.4* probe. In addition, these elevated levels are due to expression of the transgene as both tissues hybridized with the SV40 probe (Fig. 3*h* and data not shown).

Most striking of all was the intense labelling observed in the gut of transgenic embryos; little or no labelling above background was observed in the gut of non-transgenic embryos. This

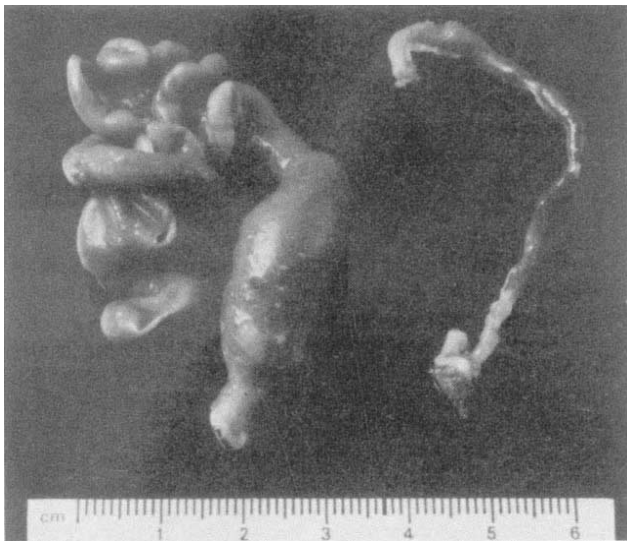


Fig. 4 Example of the megacolon phenotype from the 1975-2 lineage. Left, the dissected colon and a portion of the intestinal region of an adult mouse exhibiting profound megacolon. Right the colon of a normal adult mouse. The distention begins ~0.5 cm from the most distal region of the colon.

labelling was observed in both the 1974-3 (Fig. 3*b-e*) and 1975-2 (Fig. 3*h, i*) lineages. Hybridization with the SV40 probe confirmed that the transcripts observed in the gut were due to expression of the transgene (Fig. 3*f, h, i*). The transcripts were clearly localized to the region of mesenchyme in an evenly distributed, non-punctate pattern. No labelling above background was observed in the inner (mucosa) or outer (serosa) layers. Although it was not evident in the *in situ* hybridization experiments shown in Fig. 3, *Hox-1.4* mRNA has been detected by *in situ* analysis in the mesenchyme of the gut and lung of normal day-12.5 embryos (D. Duboule, S. Gaunt and R. Krumlauf, personal communication). Thus, the elevated expression of the *Hox-1.4* transgene probably represents overexpression of *Hox-1.4* in cells that normally express this gene, rather than ectopic expression.

The highly elevated levels of *Hox-1.4* in the developing gut of both the 1974-3 (Fig. 3*a-f*) and 1975-2 (Fig. 3*g-i*) lineages are particularly interesting because of the unexpected phenotype that develops in these mice. Our initial analysis concentrated on the 1974-3 lineage because the animals were relatively healthy and bred well. The 1975-2 lineage was quite difficult to establish. We determined that the founder male was a germ-line mosaic, transmitting the transgene to ~20% of the embryos. Only two of more than 100 weanlings generated from this founder animal carried the transgene, however. These observations suggested that transgenic pups were dying between birth and young adulthood; we have subsequently determined that they succumbed to a condition resembling congenital megacolon¹⁶. Mice with megacolon have difficulty extruding faeces through the most distal portion of their colon, resulting in a grossly distended bowel, compacted faeces and, in many cases, death. An example of this phenotype is illustrated in Fig. 4. This condition varied in severity and therefore most animals lived long enough to reproduce either on their own (occasional males) or via superovulation and embryo transfer. The original 1975-2 founder male eventually succumbed to megacolon when 11 months old.

Every transgenic animal examined in the 1975-2 lineage exhibited the megacolon phenotype. We have also observed the megacolon phenotype in two animals from the 1974-3 line and in four lines of transgenic mice carrying the 5 kb *Hox-1.4*-SV constructs. Preliminary observations on expression of the trans-

genes in one of these 5 kb lines have revealed elevated levels of *Hox-1.4* expression in the mid-gestation gut (data not shown). The megacolon phenotype varied in severity among the various lines, with the 1974-3 lineage rarely showing symptoms to one of the 5 kb *Hox-1.4*-SV lines in which all progeny carrying the transgene exhibited megacolon soon after birth and died before they were 10 days old. Multiple lines of transgenic animals exhibited a similar phenotype, indicating that the alteration in gut development was due to expression of the transgene, and not to a dominant insertional mutation.

There are several mouse mutant strains that exhibit megacolon as either homozygotes (for example, piebald lethal and lethal spotting¹⁷) or heterozygotes (dominant megacolon¹⁸). A congenital condition known as Hirschsprung's disease results in megacolon in man¹⁹. In each of these conditions the affected individuals exhibit a deficiency of myenteric ganglion cells in the colon¹⁶. These are neural crest derivatives that migrate into the developing gut to innervate the bowel¹⁶. The defect which results in megacolon may therefore be intrinsic to the neural crest cells; alternatively, the defect may lie in cells in the various layers of the developing gut that provide cues to the migrating neural crest cells. It is thus noteworthy that the *Hox-1.4* transgene is overexpressed in the mesenchymal layer of the day-12.5 embryonic gut of the transgenic mice.

It seems that the increased level of *Hox-1.4* mRNA in the transgenic embryos is due to varying levels of elevated expression of the transgene at the appropriate sites, rather than to ectopic expression. Although there could be effects of the slightly elevated levels of *Hox-1.4* mRNAs in the lung and CNS, we have not observed any obvious developmental defects. In contrast, the development of megacolon in adult mice correlates with high levels of *Hox-1.4* transcripts in the embryonic gut and constitutes the first example of a mutant phenotype associated with the expression of a mammalian homoeobox-containing gene.

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In vivo binding pattern of a *trans*-regulator of homoeotic genes in *Drosophila melanogaster*

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The specification and maintenance of the metameric pattern in *Drosophila melanogaster* is regulated by complicated gene interactions. The differential expression of the homoeotic genes of the *Antennapedia* complex (ANT-C) and *bithorax* complex (BX-C), which determine segmental identities, is partly controlled by cross-regulatory interactions of loci within the two clusters and partly by *trans*-acting factors located outside the two complexes. One of the *trans*-regulatory genes, *Polycomb* (*Pc*), acts as a repressor of the ANT-C and BX-C¹⁻⁴. Mutations of *Polycomb* result in a complete derepression of the homoeotic genes, leading to abdominal transformations of all body segments. *Polycomb* is part of a large class of *trans*-regulatory genes (*Pc*-group), estimated to comprise up to 40 loci⁵. We have raised antibodies against the

Polycomb protein, and, using an improved immunostaining technique, showed that the *Polycomb* protein binds to 60 discrete sites along the polytene chromosomes of salivary glands. These sites comprise the ANT-C and the BX-C as well as several locations of *Pc*-group genes. This is the first clear evidence for a direct interaction of *Polycomb* with homoeotic loci and other *Pc*-group genes.

The *Polycomb* locus localizes to polytene chromosome band 78 D 7,8. The DNA encompassing the gene has been cloned by a microdissection technique (R.P., J. Lauer and D. S. Hogness, manuscript in preparation). About 4 kilobase (kb) of DNA enclose the entire *Pc* locus, as shown by P-element-mediated germline transformation. *Polycomb* encodes three different transcripts. Poly(A)⁺ transcripts of 2.5 kb and 2.0 kb are very abundant early in development (0–6-h-old embryos). The 2.5 kb RNA is also transcribed during the remaining embryonic, larval and pupal stages, although at lower levels. A 1.0 kb transcript is especially abundant in late third instar larvae: in larval salivary glands it is the only *Pc* transcript found. Several complete cDNAs for the 2.5 kb transcript were isolated. Their sequence reveals an open reading frame with a coding capacity for a 390 amino-acid *Polycomb* protein of relative molecular mass (*M_r*) 44,000 (44K) (R.P. and D. S. Hogness, manuscript in preparation).

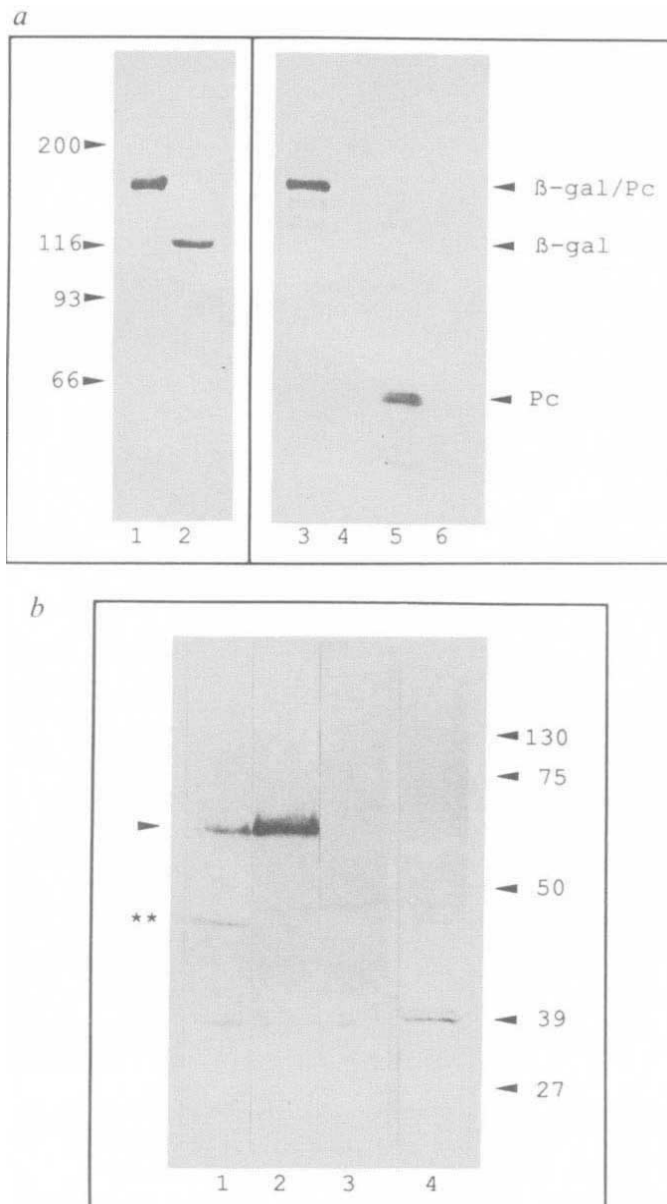
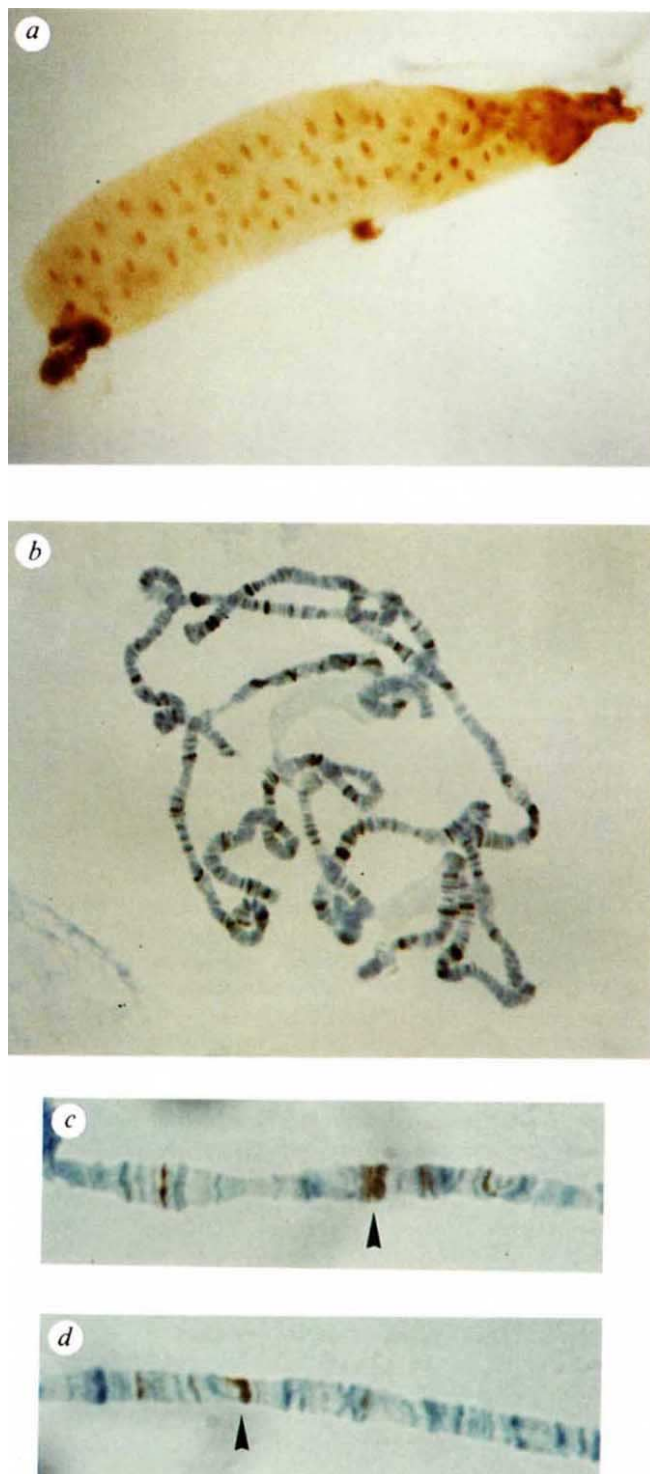


Fig. 1 Antibodies against the *Polycomb* protein. Polyclonal antibodies were raised against a lacZ-*Pc* fusion protein containing the C-terminal 199 amino acids of a *Polycomb* protein encoded by the 2.5 kb transcript. **a**, Lanes 1 and 2: partially purified β -gal-Pc and β -gal, respectively, were separated on a SDS-PAGE gel and stained with Coomassie Blue. Lanes 3 and 4: immunoblotted proteins equivalent to those in lane 1 and 2, respectively. Lane 5: a T5 expression vector (pUHE 21-3)²¹ was used to express the authentic *Pc* protein in *Escherichia coli*. Crude extracts were separated on a SDS-PAGE gel and immunoblotted. Lane 6: immunoblotted crude *E. coli* extract harbouring the T5 vector only. Size markers are indicated on the left in thousands. **b**, *Drosophila* protein extracts were separated on SDS-PAGE gels and transferred to nitrocellulose. Western blots were incubated with purified polyclonal anti-*Pc* antibodies (lanes 1–3). Lane 1: extract from 150 third instar larval salivary glands. The lower band (**) shows the smaller, salivary gland-specific *Pc* protein. The 44K *Pc* protein is also visible (arrow), resulting from contaminating fat body cells (see also Fig. 2a). Lane 2: extract from 300 wild-type embryos staged between 18–22 hours. Only the predominant 44K *Pc* protein is visible. Lane 3: extract from 300 homozygous *Pc^K*-mutants staged between 18–22 hours, showing the absence of the specific 44K *Pc* band. The *Pc^K*-mutant was found to have a 2.0 kbp deletion in the *Pc* gene (R.P., J. Lauer & D. S. Hogness, manuscript in preparation). Lane 4: same extract as in lane 2 except that the western blot was incubated with the second antibody only. Size markers are indicated on the right, in thousands. The 44K *Pc* protein and presumably also the smaller *Pc* protein migrate aberrantly on a SDS-PAGE gel compared to the calculated *M_r* deduced from the cDNA sequence.

Methods. A 920-base pair (bp) cDNA fragment was cloned into the *Bam*HI site of pUR278 (ref. 22). BMH71-18 host cells were grown and induced as described²³. The preparative purification of the 150K fusion protein was accomplished by urea extraction of the insoluble aggregate as described²³. Out of one litre of culture we obtained 20–40 mg fusion protein, which was 90% pure. Rabbits were immunized with 100 μ g fusion protein in PBS/complete Freund's adjuvant and boosted with 40 μ g antigen in PBS/incomplete Freund's adjuvant. For the purification of anti-*Pc* antibodies crude antiserum was loaded on a β -galactosidase-Affigel 15 column (equilibrated in 0.1 M Tris-Cl pH 8). The flow-through was passed over a β -galactosidase-*Pc*-Affigel 15 column (equilibrated as above). This column was washed with 0.1 M Tris-Cl pH 8, 0.5 M NaCl. Antibodies were eluted with 0.1 M glycine-Cl pH 2.5. The specificity of the anti-*Pc* antibodies was checked by western blots of *Pc* proteins produced in *E. coli* (see Fig. 1a, lanes 3–6) and to *Drosophila* protein extracts (see Fig. 1b). *Drosophila* extracts were prepared as described²⁴.

Fig. 2 Immunohistochemical localization of the Pc protein in salivary glands and on polytene chromosomes of third instar larvae. *a*, Single salivary gland showing intense staining of the large polytene nuclei. The tip of the gland shows strong staining of fat body cells. *b*, Immunostaining of squashed salivary gland chromosomes. The Pc protein is found associated with about 60 chromomeres (see Table 1) distributed over all chromosomes. No Pc protein is found in the centromere and in the nucleolus. *c*, Section of the 3rd chromosome showing the localization of the ANT-C (84 A 4,5-B 1,2; arrow). The most intense labelling is found in the distal portion of the complex (84 B 1,2). *d*, Section of the 3rd chromosome showing the association of Pc protein with the BX-C (89 E, arrow). The proximal-distal orientation of the chromosomes in *c* and *d* is from left to right.

Methods. Immunostaining of whole salivary glands. Salivary glands were dissected in PBS, 0.1% Triton X-100, fixed for 5 min in buffer A (100 mM HEPES pH 7.0, 2 mM MgSO₄, 1 mM EGTA) containing 1% formaldehyde. Glands were then washed several times with buffer A and then with BBT pH 7.0 (10 mM Tricine, 55 mM NaCl, 40 mM KCl, 7 mM MgCl₂, 5 mM CaCl₂, 20 mM glucose, 50 mM sucrose, 0.1% BSA, 0.2% Tween20). Glands were incubated for 1 h in BBT containing affinity-purified anti-Pc antibody, diluted to the desired concentration. Primary antibody incubation was followed by rinsing the glands in BBT. Then anti-rabbit IgG biotinylated antibody (preabsorbed to embryos, 1:1,000 dilution; Vector) in BBT, 1% normal serum was added for 40 min. Glands were then rinsed as above, followed by several short rinses in PBT (PBS, 0.1% Tween20). PBT was then replaced by PBT containing 1% reagent A (Avidin DH), 1% reagent B (biotinylated HRP) (both Vectastain ABC kit, Vector), which had been preincubated for 30 min. After a 40 min incubation the glands were washed again in PBT. Glands were stained with PBT, 0.005% DAB, 0.025% H₂O₂. The staining reaction was stopped by several washes in PBS. Salivary glands were mounted in 50% glycerol, PBS. Immunostaining of polytene chromosomes. Dissected salivary glands were transferred into a droplet of 3.7% formaldehyde, 1% Triton X-100, PBS pH 7.5 for 30 s. The glands were then moved in a 40 µl drop of 3.7% formaldehyde, 50% acetic acid on a coverslip for 2-3 min. Glands were squashed, the slides frozen in liquid nitrogen and the coverslip flipped off with a razorblade. The slides were rinsed in PBS, followed by BBT. The immunostaining procedure was essentially the same as for the glands. For all the incubation steps, the slides were kept in a humid atmosphere with a coverslip placed over the chromosomes. After immunostaining, chromosomes were stained for about 15 s in a 1:100 diluted Giemsa solution. After brief washing in distilled water, the chromosomes were embedded in 99.5% glycerol and immediately examined under the microscope. Photographs were taken on Kodak Ektachrome film using a Zeiss Axioplan microscope.



We have raised antibodies to the 199 C-terminal amino acids of the Polycomb protein. Polyclonal antiserum was purified and used to detect the protein in extracts of wild-type embryo (see Fig. 1b lane 2). The specificity of the antiserum is shown by the absence of the Pc protein band in western blots of extracts from mutant embryos (see Fig. 1b, lane 3). In addition, we have visualized the embryonic distribution of the Pc protein by immunostaining of whole mount embryos (B.Z. and R.P., manuscript in preparation). No immunostaining was seen in homozygous *Pc*⁻ mutant embryos (data not shown).

The expression of a specific *Pc* transcript in salivary glands of third instar larvae encouraged us to analyse this tissue for the presence of the Pc protein. Salivary glands contain large

polytene chromosomes, which greatly facilitate the analysis of gene interactions. Figure 2a shows the specific labelling of the large nuclei of a salivary gland by the anti-Pc antibodies. The salivary gland-specific 1.0 kb transcript does not have sufficient coding capacity for a 390 amino-acid protein. It encodes a smaller Pc protein, which is also specifically recognized by the antiserum (Fig. 1b, lane 1). Spreading and immunostaining of the polytene chromosomes revealed that this Pc protein is associated with approximately 60 sites (Fig. 2b and Table 1). Cytological identification of the binding sites showed that Pc is associated with loci shown to interact with *Pc* by genetic methods (see Table 2). Pc protein is abundantly found at the ANT-C (84 A 4,5-B 1,2) and at the BX-C (89 E) (Fig. 2c, d). The strong

Table 1 Pc Binding sites

X Chromosome	2nd Chromosome	3rd Chromosome	4th Chromosome
1 B	21 AB	61 C 1,2	102 D 1
2 D 1-4	22 A	62 E	102 C 1
4 C 1,2	22 C	63 A	
5 A	24 A	65 D	
5 D 1,2	25 EF	67 D	
7 B	26 F	67 F	
8 A	29 DE	68 E	
9 B 5-8	32 EF	69 C 1	
14 A	33 F	69 D 1,2	
14 F	35 C	70 A	
	36 B	82 E 1	
	38 F	84 A4,5-B 1,2	
	41 CD	84 D 1,2	
	43 C 1	84 F 1,2	
	44 A	85 E 1	
	48 A	86 C 1,2	
	48 C	88 A 1,2	
	49 E 4-7	89 B 1,2	
	51 A	89 C	
	56 BC	89 E	
	56 F6-9	90 E	
	59 EF	93 E 1-4	
	60 E1	96 A 1,2	
		98 A	
		99 EF	

Location of Pc binding sites on salivary gland polytene chromosomes of *Oregon-R* wild-type larvae. The cytological positions of the binding sites were determined by comparison of the polytene bands to the map of Bridges¹⁴.

Pc antibody staining at the positions of these two complexes may reflect the regulatory activity of *Polycomb* on the large number of homoeotic genes located in these two clusters. In addition, Pc antibody staining is associated with several cytologically identified locations of *Pc*-group genes. Major exceptions were the *esc*⁶ and the *Pcl*⁷ loci, where there was no binding of the Pc protein. A very prominently labelled site was found at 49E 4-7, the presumptive location of *Psc*⁵. Pc labelling was also found associated with sites to which a third class of genes has been mapped^{8,9}. These genes were found to interact with *Polycomb*, by either enhancing or suppressing the posterior transformations observed in *Pc* mutants. They do not cause a *Pc*-like phenotype on their own, however, and their physiological function in development is unknown.

Table 2 shows the sites of Pc antibody labelling at locations of mapped genes, previously shown by genetic analysis to interact with *Pc*. The resolution of the cytological mapping is obviously limited by the size of the chromosomal aberrations used to define the location of a gene. Because the binding sites overlap with several members of a certain class, however, it is highly probable that Pc does indeed bind and regulate the genes indicated in Table 2.

Salivary glands are ectodermally-derived accessory organs of digestion and glue secretion, developing from an invagination of the labium¹⁰. No expression of homoeotic genes in this type of tissue has been reported so far. The presence of the Pc protein, acting as a repressor, would be consistent with this result. Although the binding pattern on polytene chromosomes of salivary glands can only represent the status of this particular tissue, genetic evidence has shown that Pc interacts with the same genes in other ectodermally-derived tissues^{1-4,11-13}. This suggests that the pattern seen in salivary glands is unlikely to be completely different from that expected for other types of tissue. The terminal differentiation of larval salivary glands, which histolyse at metamorphosis, might indicate a default setting of *Polycomb*, keeping all homoeotic genes in a repressed

Table 2 Pc Binding to cytologically mapped regulatory genes

	Cytology*	Binding sites of Pc
Homoeotic genes		
ANT-C	84 A 4,5-B 1,2	84 A 4,5-B 1,2
BX-C	89 E	89 E
<i>Pc</i> -group genes		
Additional sex combs (<i>Asx</i>)	51 AB	51 A
Extra denticles (<i>exd</i>)	13 F 1-14 B 1	14 A
Polycombeotic	67 F	67 F
Polyhomoeotic (<i>ph</i>)	2 D 2-3	2 D 1-4
Posterior sex combs (<i>Psc</i>)	49 EF	49 E 4-7
Sex combs on midleg (<i>Scm</i>)	85 EF	85 E 1
Super sex combs (<i>sxc</i>)	41 C	41 C
<i>Pc</i> -interacting genes		
Enhancer of (<i>Pc</i>)	48 A	48 A
Suppressor of (<i>Pc</i>) ¹	21 AB	21 AB
Suppressor of (<i>Pc</i>) ^{2,3}	35 CD-38 B	35C+36 B
Suppressor of (<i>Pc</i>) (<i>Brista</i> ^{MP})	60 D 14-E 2	60 E 1

Location of selected Pc binding sites that overlap with cytologically mapped genes with which Pc is interacting. Cytological positions (*) were taken from the following references: ANT-C (ref. 15); BX-C (ref. 1); *Asx*, *Psc* and *Scm* (ref. 5); *exd* (ref. 16); *polycombeotic* (A. Shearn, personal communication); *ph* (ref. 17); *sxc* (ref. 18); *E(Pc)* (ref. 8); *Su(Pc)*^{1,2,3} (ref. 9); *Su(Pc)(Brista*^{MP}) (refs 9, 19). 48A is also the location of the *engrailed* gene (ref. 20), as Pc was shown to interact with *engrailed* also (G. Morata, personal communication).

state. At earlier developmental stages *Polycomb* might, with the help of different sets of *Pc*-like genes, repress homoeotic genes in a segment- and tissue-specific way.

This work demonstrates the first complete *in vivo* chromosome-binding pattern of an important regulatory factor involved in metamer development. We have shown that the Pc protein binds to genes shown genetically to interact with *Pc*. In addition we have identified the cytological location of other target genes of *Pc*. It will be interesting to define the integration of *Polycomb* in this complex regulatory network and to determine whether it acts as a negative regulator of the ANT-C and BX-C, but as an activator of other target genes. In addition it will be necessary to identify how *Pc* exerts its function at the molecular level: whether it regulates genes by acting as a DNA sequence-specific binding factor, or by recognizing other proteins (for example other *Pc*-group proteins) and interacting as a regulatory complex. To unravel the molecular mechanisms generating pattern formation in *Drosophila* it is necessary to identify as many of the components participating as possible. The use of cytology to study the complicated hierarchical gene interactions involved greatly facilitates this task. Moreover, the technique used here should prove valuable in the identification of target genes for other regulatory factors in *Drosophila* and, potentially, other regulatory elements from different biological systems that can be expressed in *Drosophila*.

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Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate

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Granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) belong to a family of glycoprotein growth factors required for the survival, growth and differentiation of haematopoietic precursors and which affect the function of circulating mature cells. They are produced by resting or stimulated stromal cells of the haematopoietic micro-environment (fibroblasts and endothelium) and by immunocompetent cells (T cells and monocytes/macrophages)^{1,2}. The action of these CSF molecules was thought to be restricted to cells of haematopoietic origin. Here, we report that G-CSF and GM-CSF influence the migration and proliferation of human endothelial cells suggesting that these molecules may act as regulatory signals outside the haematopoietic system.

When different preparations of human recombinant G-CSF and GM-CSF were tested on human endothelial cells (HEC), a marked increase of migration across polycarbonate filters and incorporation in DNA of [³H-methyl]thymidine ([³H]thymidine) was observed (Fig. 1). Coating with gelatin of polycarbonate filters and plastic wells was necessary to observe HEC migration and [³H]thymidine uptake in response to G-CSF and GM-CSF. Both factors acted in a dose-dependent way. The maximal response elicited by G-CSF and GM-CSF was somewhat lower in all experiments than that elicited by the optimal concentration of fibrinogen used as a chemoattractant in these studies⁶ or by human recombinant basic fibroblast growth factor⁷ used as growth factor (Table 1). HEC grown for 48 h in 1% fetal calf serum (FCS) with G-CSF or GM-CSF entered the S-phase within 8 h, measured by the incorporation of [³H]thymidine and maximal incorporation occurred between 12 and 16 h, with lower values thereafter (data not shown). Increased DNA synthesis is coupled to a true HEC growth, with a two- to threefold increase in cell numbers after six days of culture with 140 and 250 pM G-CSF and GM-CSF, respectively (three experiments).

Cell proliferation is associated with the expression of a set of genes including *c-myc* and *c-fos*; *c-fos* is activated in response

Table 1 The G-CSF and GM-CSF molecules are active in inducing HEC migration and proliferation

Agent	HEC migration (number of cells)	[³ H]thymidine incorporation (c.p.m. × 10 ³)
Medium	44 ± 8	1.95 ± 0.86
IL-5 (1:10 ³ dilution)	49 ± 6	ND
IL-1 (50 ng ml ⁻¹)	55 ± 8	ND
G-CSF (200 pM)	72 ± 1*	17.38 ± 3.91*
Boiled G-CSF	38 ± 9	3.21 ± 1.36
G-CSF + polymixin	79 ± 12*	18.31 ± 3.71*
G-CSF + anti-G-CSF	44 ± 5	4.12 ± 2.10
G-CSF + irrelevant IgG	82 ± 14*	16.12 ± 3.12*
Fibrinogen (1 mg ml ⁻¹)	132 ± 12*	ND
Fibrinogen + anti-G-CSF	129 ± 7*	ND
GM-CSF (250 pM)	85 ± 7*	15.27 ± 3.81*
Boiled GM-CSF	37 ± 6	2.34 ± 1.23
GM-CSF + polymixin	79 ± 9*	15.34 ± 2.81*
GM-CSF + anti-GM-CSF	45 ± 6	3.51 ± 0.88
GM-CSF + irrelevant IgG	86 ± 16*	14.67 ± 1.45*
Fibrinogen (1 mg ml ⁻¹)	103 ± 12*	ND
Fibrinogen + anti-GM-CSF	104 ± 11	ND
Basic FGF (10 ng ml ⁻¹)	ND	39.12 ± 4.56*

Recombinant molecules were heat-inactivated by boiling for 10 min. Polymixin B (Sigma) was used at a concentration of 10 µg ml⁻¹. The mouse anti-human-G-CSF monoclonal antibody (mAb) 75A was from K. Welte and C. Bordinon at a concentration of 8.2 mg ml⁻¹ and a neutralizing activity of 33 U µg⁻¹. G-CSF (5 ng ml⁻¹) was preincubated with mAb (38 µg ml⁻¹) at room temperature for 40 min and then tested in a migration and [³H]thymidine incorporation assay. Two different anti-GM-CSF antibody preparations were used in these experiments. The anti-human GM-CSF mAb used in migration experiments was from Dr Kurlle. GM-CSF (5 ng ml⁻¹) was preincubated with the anti-GM-CSF mAb (1.25 µg ml⁻¹) for 40 min at room temperature and then tested in the migration assay. A polyclonal rabbit anti-GM-CSF antibody (1:1,000 dilution) was used in proliferation experiments. The OKT11 mAb (Ortho) or mouse normal IgG were used as irrelevant IgG in these experiments. Human recombinant IL-1 was from Sclavo, Siena, Italy. Fibrinogen (from Dr E. Dejana) and human recombinant basic fibroblast growth factor (FGF) (Amersham) were used as reference agents active on HEC migration⁶ and proliferation⁷. For basic FGF-induced [³H]thymidine incorporation, the experiment was performed as described in the Fig. 1 legend, except that an incubation time of 48 h with the growth factor (optimal for FGF) was used. ND, not determined.

* $P < 0.05$ versus medium control.

to chemoattractants⁸, including G-CSF and GM-CSF⁹. Using northern analysis we detected a marked induction of *c-fos* mRNA in HEC (peak at 2 h) in the presence of cycloheximide (a superinducer of *c-fos* mRNA¹⁰).

In an attempt to identify the G-CSF and GM-CSF receptors on HEC that are responsible for the unexpected responses evoked by these cytokines in these non-haematopoietic cells, we studied the binding of ¹²⁵I-labelled molecules to HEC. Figure 2 shows representative experiments of the three performed for each cytokine. High-affinity binding sites were identified on HEC. The receptor number on the cell surface was estimated to be 356 ± 123 and 423 ± 187 with a dissociation constant (K_d) of 21 ± 8 pM and 54 ± 31 pM for G-CSF and GM-CSF, respectively (mean ± s.d. of three experiments). These values are of the same order of magnitude as those estimated for myelocytic or monocytic cells¹¹⁻¹³.

The unexpected effects of G-CSF and GM-CSF on the migration and proliferation of HEC could be due to contaminants present in the recombinant CSF preparations. The first indication that G-CSF and GM-CSF were indeed responsible for these activities was provided by the cytokine specificity of these effects. Monocytic-CSF (Fig. 1a) and interleukin 5 (IL-5) (Table 1), an

Fig. 1 Induction of HEC migration and proliferation by G-CSF (a, ●), monocytic-CSF (M-CSF, a, ▲) and GM-CSF (b, ●).

Methods. From the human umbilical vein, HEC (III-VIII passage) were cultured as described³. The cells (2×10^4 per well, 24 well plates precoated with 0.05% gelatin for 24 h) were grown for 24–36 h in M199 with 20% FCS and then starved for 48 h in 1% FCS with 0.5% bovine serum albumin (BSA, fraction V, Sigma). Medium was then removed and replaced with control medium containing 2% FCS with or without recombinant G-CSF (Dr T. Okabe, 7.8×10^7 U ml⁻¹; or Amgen, 1.5×10^8 U mg⁻¹), GM-CSF (Genetics

Institute; or Behringwerke; or Immunex, 10^7 U mg⁻¹) and M-CSF (Cetus, 4×10^7 U mg⁻¹). The cells were incubated for 11 h at 37 °C, [³H-methyl]thymidine (0.5 µCi; 30 Ci mmol⁻¹, Amersham) was added and incubation continued for 1 hour. Medium was removed, cells were washed twice and detached by trypsinization. DNA was precipitated with 10% cold trichloroacetic acid and collected on 0.2 µm Gelman filters⁴. Radioactivity was measured by liquid scintillation counting. Results presented are the mean $\pm$ s.d. of three experiments in triplicate. Chemotaxis experiments were performed with a 48-well modified Boyden chamber technique^{5,6}. Polycarbonate filters (5 µm pore size, polystyrene-pyrrolidone-free) were soaked in 0.5 M acetic acid, washed with PBS, incubated for 24 h in 0.01% gelatin (Sigma) and air-dried. G-CSF, GM-CSF and M-CSF (25 µl) in M199 containing 0.2% BSA were seeded in the lower compartments and 50 µl of HEC (2×10^6 ml⁻¹) were added in the upper ones. After 6 h incubation at 37 °C, the upper surface of the filter was scraped with a rubber policeman. The filters were fixed and stained, and five oil immersion fields (lower surface) were counted after coding the samples. Results are presented as mean $\pm$ s.d. migrated cells (statistical significance assessed) by Dunnet's test. Results presented are from individual experiments out of nine (GM-CSF) or three (G-CSF, M-CSF).

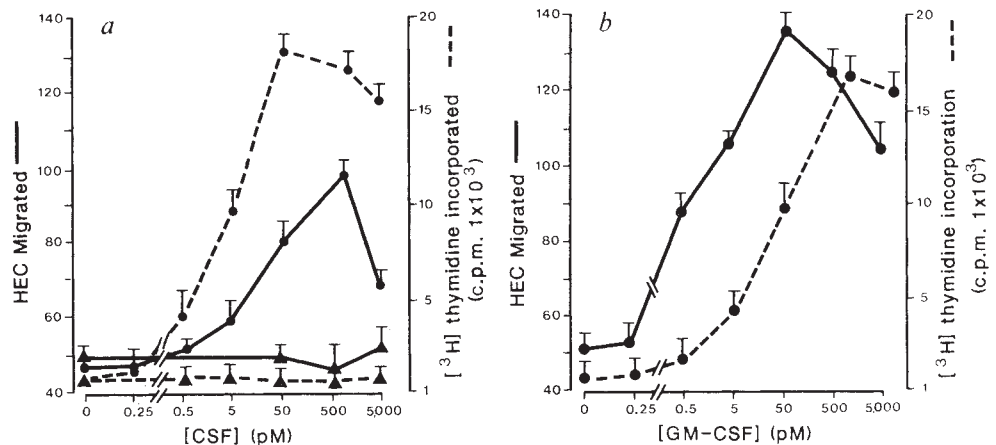
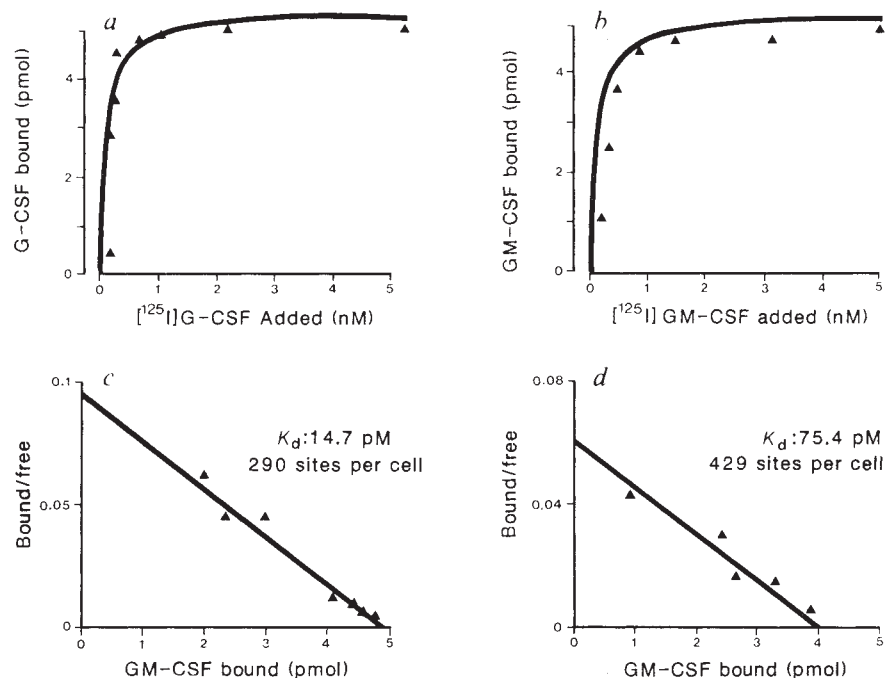


Fig. 2 G-CSF and GM-CSF receptors on HEC. a, b, Saturation curve at equilibrium; c, d, Scatchard representation of the specific binding.

Methods. Binding experiments have been performed by using [¹²⁵I] G-CSF and [¹²⁵I] GM-CSF. G-CSF and GM-CSF (30 µg per 20 µl borate buffer 0.1 M, pH 8) were iodinated using Bolton-Hunter reagent (Amersham; 0.5 mCi for each cytokine). Labelled cytokines were passed through Sephadex G-25. The specific activity of the radiolabelled G-CSF (6 ng per 10^5 c.p.m.) and GM-CSF (7.4 ng per 10^5 c.p.m.) was assessed by measuring protein concentration. Iodinated cytokines are active on HEC in terms of migration and proliferation. HEC were detached from the plastic flasks by incubation for 10 min at 37 °C in PBS containing 1 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid and then washed twice in medium 199 containing 25 mM (4-(2-hydroxyethyl)-1-piperazinoethanesulphonic acid, pH 7.4 and 0.2% BSA (binding buffer). Cells (4×10^6 ; 95–97% viable) in 0.4 ml of binding buffer containing different concentrations of labelled G-CSF and GM-CSF, with or without 100-fold excess of unlabelled cytokines, were incubated for 2 h at 23 °C. In preliminary experiments, binding at 23 °C required 2 h to reach the equilibrium for both cytokines. The reaction was stopped by centrifugation of the cells, which were overlaid onto 0.8 ml of cold binding buffer containing 75% FCS¹¹ and the radioactivity in the pellet was determined. Specific binding was determined as the amount of binding inhibited by 100-fold excess of the unlabelled G-CSF or GM-CSF. Binding data were analysed by nonlinear least squares curve fitting with ENZFITTER program (IBM). Data shown are representative of three different experiments done in triplicate.



eosinophil CSF¹⁴, had no effect on the migration and proliferation of HEC. Similarly, IL-1, a molecule active on haematopoietic progenitors¹⁵ and with profound effects on HEC function¹⁶, did not induce HEC migration (Table 1). Thus, the capacity to elicit a migratory and proliferative response in HEC is not shared by all recombinant molecules active on haematopoietic precursors, even when these cytokines, like IL-1, profoundly affect other HEC functions. Polymixin B which

inhibits bacterial lipopolysaccharides, did not affect the activity of G-CSF and GM-CSF (Table 1). Boiling abolished the activity of G-CSF and GM-CSF (Table 1). Anti-G-CSF and anti-GM-CSF antibodies abolished the induction of HEC functions (Table 1). Irrelevant control antibodies were inactive and the inhibitory effect of specific antibodies did not seem to be due to non-specific effects, as there was no effect on the activity of fibrinogen. We also tested the possibility that a non-HEC cell

Table 2 Checkerboard analysis of induction of HEC migration by GM-CSF

Experiment 1		Above		
Below	Medium	0.5 pM GM-CSF	5 pM GM-CSF	50 pM GM-CSF
Medium	74 ± 3	75 ± 2	78 ± 8	82 ± 4
GM-CSF (pM)				
0.5	87 ± 6	77 ± 16	88 ± 1	93 ± 4
5	106 ± 3*	96 ± 6*	95 ± 1*	100 ± 6*
50	110 ± 2*	97 ± 1	115 ± 12*	101 ± 11*
Experiment 2		Above		
Below	Medium	5 pM GM-CSF	50 pM GM-CSF	500 pM GM-CSF
Medium	46 ± 4	35 ± 3	39 ± 7	39 ± 3
GM-CSF (pM)				
5	75 ± 7*	64 ± 7	51 ± 4	42 ± 2
50	79 ± 7*	72 ± 2*	69 ± 8	54 ± 7
500	128 ± 7*	92 ± 5*	77 ± 1*	70 ± 2*

Different concentrations of GM-CSF were placed in the upper and/or lower compartments of the chemotaxis chamber. Results are expressed as the number of migrated HEC (mean ± s.d. of three samples for each experimental point).

* $P < 0.05$ versus medium control.

contaminant (that is, monocytes), might directly or indirectly account for the actions of G-CSF and GM-CSF on HEC. HEC cultures consisted of homogeneous cells with typical cobblestone morphology, having (>99%) granular deposits of factor VIII antigen, as well as H-lectin antigen and class I, but not class II, histocompatibility antigens on the surface. We could not detect cells expressing CD11b, CD14 or CD3 with flow cytometry. Treatment with anti-CD11b or anti-CD3 and complement did not affect the responses of HEC to the two CSF. The human endothelial cell line EAhy926 (refs 17–19) migrated and expressed *c-fos* mRNA after stimulation with G-CSF and GM-CSF and had specific receptors for these molecules (data not shown). Collectively, these data showed that HEC do indeed have receptors for and respond to G-CSF and GM-CSF.

In checkerboard experiments⁶, maximal induction of migration across filters occurred in the presence of a positive concentration gradient between the lower and upper compartments of the chamber (Table 2). With equal concentrations of GM-CSF above and below the filter, a smaller, but significant enhancement of HEC migration was observed, suggesting that there is a chemokinetic component, in addition to chemotaxis, in the action of GM-CSF on HEC locomotion. G-CSF and GM-CSF also induced migration when HEC had adhered to the filters before the assay, excluding the possibility that these cytokines were merely affecting the speed of adhesion of HEC to filters.

The results presented here demonstrate that G-CSF and GM-CSF induce migration and proliferation of HEC, in what we believe is the first demonstration of activity of these molecules on non-haematopoietic cells. Other examples, of leukocyte-specific non-pleiotropic cytokines acting on non-hematopoietic elements, are monocytic-CSF acting on trophoblast cells and IL-2 acting on glial cells^{20,21}. Our observation is interesting in the light of the many similarities between myelomonocytic cells and endothelium, illustrated by previously widespread use of the expression 'reticuloendothelial system'.

G-CSF and GM-CSF are produced by various cell types including stimulated lymphocytes, activated macrophages^{1,2} and endothelial cells exposed to monokines^{22,23}. IL-1 has been reported to induce proliferation of vascular elements²⁴ and *c-fos* expression in HEC²⁵. The functionally related monokine tumour necrosis factor, however induces the migration of endothelial

cells²⁶, but inhibits their proliferation²⁷. These monokines do, however, elicit a complex set of responses in HEC, which include CSF production. For example, IL-1-induced proliferation of vascular smooth muscle is masked by the concomitant induction of growth inhibitory prostaglandins²⁴. Proliferation of endothelial cells and neovascularization occur at sites of cell-mediated immunity and inflammation²⁸ and locally produced G-CSF and GM-CSF may be one of the factors involved in the fine regulation of the proliferation and migration of endothelium. Survival and growth of endothelium in bone marrow stroma is essential for the haematopoietic microenvironment and haematopoiesis. The capacity of appropriately stimulated HEC to produce G-CSF and GM-CSF suggests that these molecules may participate in autocrine regulation of function.

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Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin

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Peptidyl-prolyl *cis-trans* isomerase (PPIase) catalyses the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides and has been shown to accelerate the refolding of several proteins *in vitro*^{1–4}. Its activity has been detected in yeast, insects and *Escherichia coli* as well as in mammals, and it is thought to be essential for protein folding during protein synthesis in the cell¹. We purified PPIase from pig kidney and found that its amino-acid sequence is identical to that reported for bovine cyclophilin, a protein known to bind the immunosuppressive drug, cyclosporin A (ref. 5). To investigate the functional relationship between

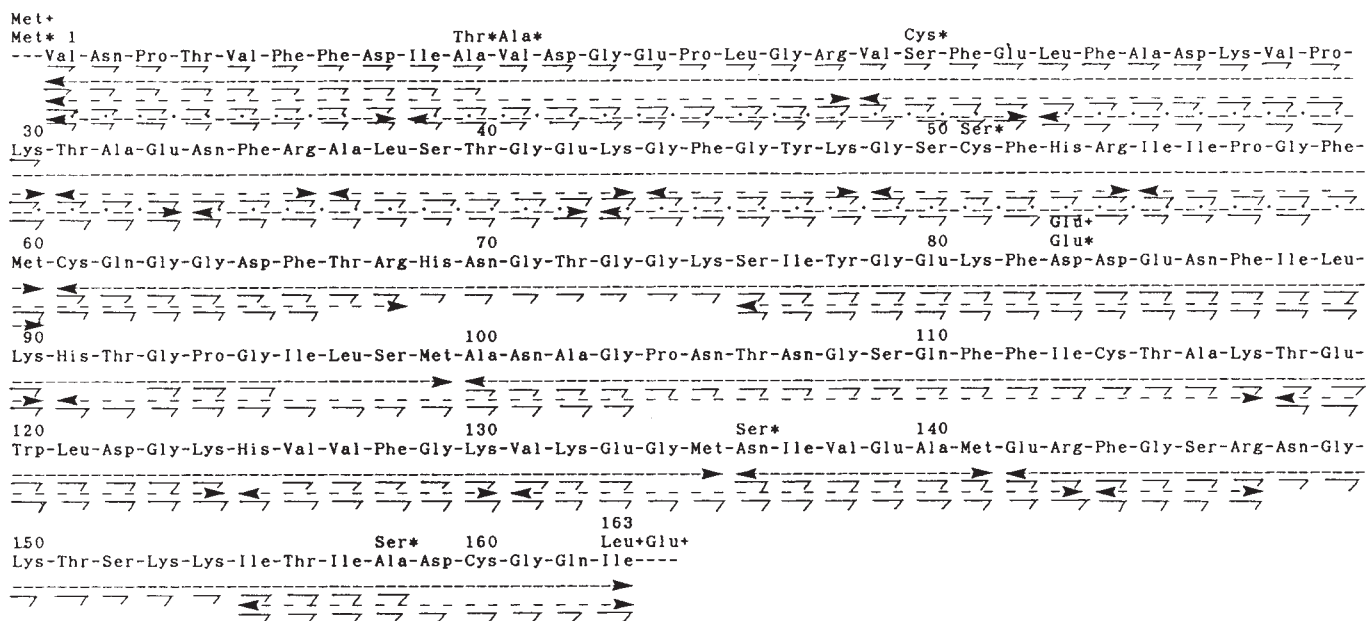


Fig. 1 Amino-acid sequence of form *a* of pig PPIase. The peptides from which the sequence was derived are as follow: ---, tryptic peptides; - - -, cyanogen bromide peptides; and — — —, *Staphylococcus aureus* V8 protease peptides of the amino-terminal cyanogen bromide peptide. After digestion of the native protein with either trypsin or cyanogen bromide, the peptides containing Cys 61 and Cys 160 purified as single peaks which could be separated further (to give the tryptic peptides 55–68 and 155–163, or the cyanogen bromide peptides 61–99 and 142–163) on reduction and carboxymethylation, indicating the existence of a disulphide bond. In contrast the peptides containing Cys 51 and Cys 114 purified separately, even without alkylation and reduction. The arrows at the amino terminus and under each peptide line indicate sequenator analyses of the intact form *a* and the peptides obtained. Differences between the pig and the rat, and/or the human sequences are indicated above the corresponding residues and designated by * for rat and by + for human. The amino-acid sequences of rat and human were deduced from their cDNA sequences^{7,8}.

Methods. The amino-acid sequence analysis was carried out by automated Edman degradation using Applied Biosystems 477A protein sequencer equipped with a model 120A on-line PTH analyser. The native protein (20–100 µg) was subjected to separate digestions with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, and cyanogen bromide. The digests were separated on an Aquapore RP-300 column or on a Spheri-SRP-18 column with the use of 130A separation system (Applied Biosystems). The columns were eluted at a flow rate of 200 µl min⁻¹ with a linear gradient of acetonitrile (0–100%) containing 0.1% trifluoroacetic acid or 0.1% heptafluorobutylic acid for 45 min. The amino-terminal cyanogen bromide peptide was further digested with *Staphylococcus aureus* V8 protease. Amino-acid analysis was carried out by Beckman 6300 E amino-acid analyser after hydrolysis with 6 M HCl for 24 h.

PPIase and cyclophilin we examined the effect of cyclosporin A on PPIase activity and found that it was inhibitory. Thus we propose that the peptidyl-prolyl *cis-trans* isomerizing activity of PPIase may be involved in events, such as those occurring early in T-cell activation, that are suppressed by cyclosporin A.

We detected PPIase activity, by the assay described in the Fig. 2 legend, in a crude ammonium sulphate extract prepared from pig kidney¹. PPIase was purified further as described in Table 1, and after a final step of purification by reversed-phase column chromatography, was separated into two forms (*a* and *b*). These isoforms have an apparently identical relative molecular mass (M_r) of 18,000 and very similar amino-acid compositions (data not shown). Although the observed relative molecular mass is somewhat at variance with the value previously reported ($M_r = 14,000$)³ we were not able to detect any other major component with PPIase activity during the purification. Exposure to a high concentration of acetonitrile during the reversed-phase column chromatography decreased but did not eliminate the specific activity detected (Table 1).

The two isoforms *a* and *b* were present in a ratio of 53:47 and their specific PPIase activities were indistinguishable. A comparison of their tryptic peptide maps indicated that the structural difference between the two isoforms resides in the amino-terminal peptides (data not shown). The first 30 amino-acid residues of form *a* were unambiguously determined by Edman degradation (see Fig. 1) but the amino terminus of form *b* could not be sequenced using this method.

Sequence analysis of form *a* reveals a total of 163 amino-acid residues and predicts a relative molecular mass of 17,735, in good agreement with the estimate by SDS-gel electrophoresis. Peptide mapping data indicate the existence of a disulphide bridge between Cys 61 and Cys 160, although we cannot exclude the possibility that this was formed *in vitro*, as we did not use anti-oxidant during the purification procedure. The remaining two cysteine residues, Cys 51 and Cys 114 (see Fig. 1 legend) appear to have free sulphhydryl groups.

On searching for homologous sequences in the National Biomedical Research Foundation database, we found that the sequence of pig PPIase is identical to that of bovine cyclophilin. Cyclophilin is known to bind specifically to cyclosporin A (CsA), a cyclic undecapeptide of fungal origin, that is a potent immunosuppressant used to prevent rejection on transplantation of organs such as kidney, heart, bone marrow and liver^{5,6}. Cyclophilin is a soluble cytosolic protein and is present in high concentrations (0.1–0.4% of total cytosolic proteins) in many mammalian tissues^{5,6} and in nearly all organisms^{5–7}. Two isoforms have been isolated for bovine cyclophilin and are expected to have very similar molecular structures⁶.

The amino-acid sequence of pig PPIase (and bovine cyclophilin) is 96% identical to the published sequences for rat⁷ and human⁸ cyclophilin, and about 60% identical to the recently published sequence for *Neurospora crassa*⁹, indicating a high degree of conservation across species. PPIase was suggested to be present on ribosomal particles¹ and it is interesting that

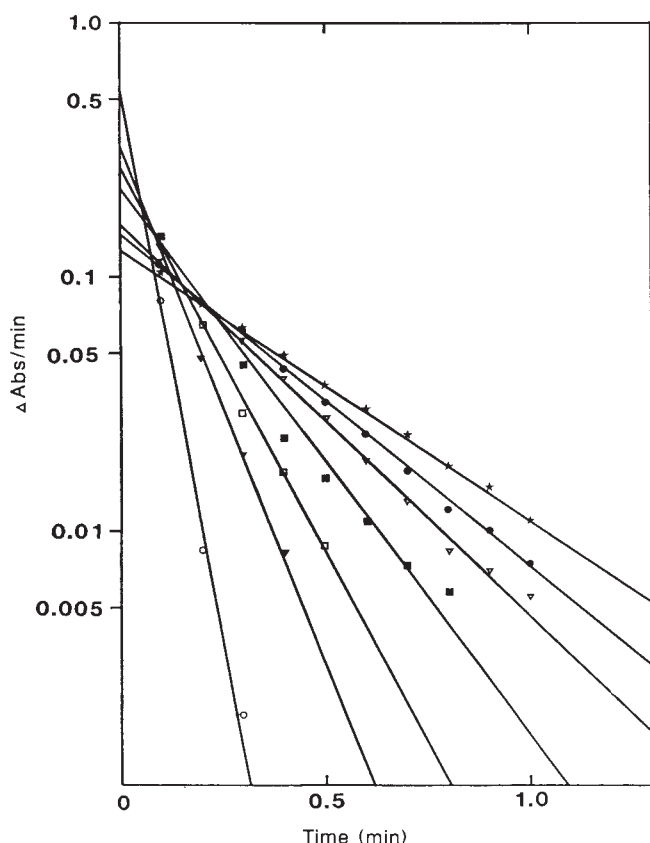


Table 1 Purification of pig kidney PPIase

Purification step	Total protein (mg)	Total activity (arbitrary units)	Specific activity (arbitrary units mg ⁻¹)	Activity recovery (%)
Ammonium sulphate fraction	7,322	183,050	25	100
DEAE-Sephacel chromatography	699	90,222	129	49
Sephacryl S-200 gel filtration	67	63,784	952	35
Sephadex G-75 gel filtration	6.7	37,218	5,555	20
TSK-phenyl 5PW-RP chromatography				
form a	1.4	1,417	1,012	1
form b	1.2	1,368	1,146	1

The ammonium sulphate extract containing PPIase activity was prepared from 690 g of pig kidney cortex by the method of Fischer *et al.*¹. The extract was dialysed against 0.01 M Tris-HCl buffer, pH 8.0, containing 0.05% NaN₃ and applied to a DEAE-Sephacel column (2.5 cm ID×35 cm). PPIase activity was measured in a coupled assay with chymotrypsin by a modified method of Fischer *et al.*¹ in which the oligopeptide *N*-succinyl-Ala-Ala-Pro-Phe-4-methyl-coumaryl-7-amide (MCA) (Peptide Institute Inc., Osaka, Japan) was used as the substrate instead of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide⁴ or glutaryl-Ala-Ala-Pro-Phe-4-nitroanilide¹. The eluate rich in PPIase activity passed through the column and was obtained as a single peak, which was concentrated by ammonium sulphate fractionation and separated on a Sephacryl S-200 column (2.5 cm ID×90 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.05% NaN₃. A major PPIase activity was found in the last peak and this fraction was rechromatographed on a Sephadex G-75 column (2.5 cm ID×90 cm). Finally, PPIase was purified by reversed-phase chromatography on a TSK-phenyl 5PW-RP column with a linear gradient of acetonitrile (0–70%) containing 0.1% TFA, and eluted as two peaks at about 40% of acetonitrile concentration. Although the specific activity of PPIase was decreased by one-fifth after the reversed-phase chromatography, it was associated with only the peaks of *M_r* = 18,000.

Fig. 2 Inhibition of PPIase activity by CsA. The kinetics of *cis-trans* isomerization of *N*-succinyl-Ala-Ala-Pro-Phe-MCA was monitored at 360 nm in a coupled assay with chymotrypsin cleavage. The MCA moiety can only be cleaved off by chymotrypsin when the preceding Ala-Pro peptide bond is in the *trans* conformation¹. At the equilibrium about 12% of the peptide has a *cis* Ala-Pro bond. In the presence of a high chymotrypsin concentration, hydrolysis of the *trans* peptide occurs within a few seconds, but cleavage of the *cis* peptide is limited in rate by the *cis* → *trans* isomerization of the Ala-Pro bond. The *trans* peptide was cleaved within the deadtime and the kinetic data of this phase is not shown. The rate of *cis* → *trans* isomerization of *N*-succinyl-Ala-Ala-Pro-Phe-MCA, represented as the absorbance change at a given time (ΔAbs/min) is plotted against the proceeding times (min) in semi-logarithmic plots. CsA clearly affected the rate of the isomerization catalysed by PPIase: ○, without CsA; ▼, 0.04 μg; □, 0.08 μg; ■, 0.16 μg; ▽, 0.4 μg; ●, 1 μg and ★, without PPIase.

Methods. Aliquots (2.0 ml) of 0.035 M HEPES-buffer containing 5 mM 2-mercaptoethanol, pH 7.8, were incubated at 25 °C for 10 min in the spectrophotometer cell (Spectrophotometer U-3210 with temperature controller SPR-7 and cell stirrer, Hitachi) before adding 50 μl 1.68 mM *N*-succinyl-Ala-Ala-Pro-Phe-MCA in the same buffer. Samples were equilibrated for 1 min. The PPIase fraction (0.27 μg) was incubated with the substrate for 30 s and the reaction was started by adding 20 μl 0.76 mM of chymotrypsin (sigma). The effect of CsA on PPIase activity was examined by mixing CsA with PPIase for 1 min before incubating with the substrate. CsA was kindly provided by Dr Y. Yamakawa of the National Institute of Health, Japan and was dissolved in saline containing 7% ethanol and 2.85% Tween 80 before use (this solvent did not affect the PPIase activity measured in these experiments). The rate of isomerization at a given time was calculated by using a rate assay program provided for Spectrophotometer U-3210.

cyclophilin was found to be localized in both the mitochondria and the cytosol of *Neurospora crassa*⁹.

The CsA-binding activity of cyclophilin is sulphhydryl dependent⁵ and the activity of PPIase is also affected by sulphhydryl blocking reagents¹, suggesting that CsA binding may modulate PPIase activity. We therefore examined the effect of CsA and found that it blocked PPIase activity (Fig. 2). Activity was almost completely inhibited in the presence of CsA at 3×10^{-7} M, a concentration that is consistent with the dissociation constant of CsA to cyclophilin (2×10^{-7} M; ref. 5). These results, and the observation that both PPIase activity and the CsA-binding activity of cyclophilin are heat-sensitive at around 50 °C, and abolished by incubation with trypsin but not with chymotrypsin^{1,6}, support, on functional grounds, the identity between PPIase and cyclophilin.

On the basis of this identity, we propose that CsA may mediate some of its effects, such as the inhibition of interleukin-2 and γ -interferon production during T-lymphocyte activation, via its inhibitory action on PPIase. We also propose that PPIase may act, not only as an enzyme that facilitates folding during protein synthesis, but as a fundamental modulator in various intracellular signal transduction processes, though *cis-trans* isomerization of the partner molecules. The ubiquitous presence of PPIase, coupled with what we know of the effects of CsA-binding, may be indicative of its significance in such biochemical processes.

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Cyclophilin and peptidyl-prolyl *cis-trans* isomerase are probably identical proteins

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The enzyme peptidyl-prolyl *cis-trans* isomerase (PPIase) was recently discovered in mammalian tissues and purified from porcine kidney¹. It catalyses the slow *cis-trans* isomerization of proline peptide (Xaa-Pro) bonds in oligopeptides and accelerates slow, rate-limiting steps in the folding of several proteins²⁻⁵. Here, we report the N-terminal sequence of PPIase together with further chemical and enzymatic properties. The results indicate that this enzyme is probably identical to cyclophilin, a recently discovered mammalian protein which binds tightly to cyclosporin A (CsA). Cyclophilin is thought to be linked to the immunosuppressive action of CsA⁶⁻¹¹. The first 38 amino-acid residues of porcine PPIase and of bovine cyclophilin are identical and the two proteins both have a relative molecular mass of about 17,000 (ref. 7). The catalysis of prolyl isomerization in oligopeptides and of protein folding by PPIase are strongly inhibited in the presence of low levels of CsA. The activities of both PPIase and cyclophilin depend on a single sulphhydryl group. At present it is unknown whether the inhibition of prolyl isomerase activity is related with the immunosuppressive action of CsA.

PPIase from pig kidney was subjected to automated N-terminal sequence analysis. The first 38 amino-acid residues of porcine PPIase are shown in Table 1. The sequence is identical to the N-terminal sequences of cyclophilin from human spleen and from bovine thymus^{7,10}. An analysis^{12,13} of the fluorescence and of the absorbance spectra of PPIase yields a content of one

tryptophan and two tyrosine residues per PPIase molecule, which agrees with the respective values found for the cyclophilins^{7,10}. Porcine PPIase and bovine cyclophilin⁷ show the same apparent relative molecular mass (M_r) of 17,000 (17K). From the sequence analysis the actual M_r is 17,737, corresponding to 163 residues⁷.

Cyclophilin forms very tight stoichiometric complexes with CsA. Values in the range of 5–200 nM were estimated for the respective dissociation constants^{6,7}. CsA binds efficiently to PPIase as well. In the presence of CsA both catalysis of *cis-trans* isomerization of a proline-containing oligopeptide and the acceleration of protein folding are inhibited. The inhibition of the activity towards the peptide Suc-Ala-Ala-Pro-Phe-4-nitroanilide is shown in Fig. 1a. The rate of *cis* → *trans* isomerization of the Ala-Pro bond is increased from $k = 7.4 \times 10^{-3} \text{ s}^{-1}$ (in the absence of PPIase) to $k > 1 \text{ s}^{-1}$ in the presence of 0.19 μM PPIase. Addition of 7 μM CsA abolishes the accelerating effect of PPIase and decreases the reaction rate to a value ($k = 9.0 \times 10^{-3} \text{ s}^{-1}$) that is similar to the uncatalysed isomerization. The interaction is reversible: diluting out CsA by 2,000-fold restores PPIase activity. Figure 1b shows the decrease of PPIase activity with increasing concentrations of CsA. The resulting inhibition constant ($K_i = 2.6 \text{ nM}$) agrees fairly well with the lower limit given for the dissociation constant ($K_d = 5\text{--}200 \text{ nM}$) of the cyclophilin/CsA complex determined under different conditions⁷.

The catalysis of slow protein folding reactions by PPIase is inhibited in the presence of CsA as well (Fig. 2). Slow refolding of ribonuclease T1 is strongly accelerated in the presence of 0.35 μM PPIase. Addition of 1.0 μM CsA inhibits catalysis of folding completely and a >95% inhibition of the PPIase activity towards folding of RNase T1 is found already in the presence of 0.5 μM CsA.

Both the binding of CsA to cyclophilin⁸ and the catalysis of proline isomerization by PPIase¹ are sulphhydryl dependent. One sulphhydryl group of PPIase is specifically protected against modification with *p*(hydroxymercuri)-benzoate (*p*HMB) when CsA is bound to the enzyme. In the absence of CsA the modification reaction is triphasic. After a fast reaction with *p*HMB two slow phases are observed in which two and one sulphhydryl group per PPIase molecule, respectively, are modified (Fig. 3a). The slowest phase is not observed in the presence of CsA. Apparently binding of CsA protects the most

Table 1 Determination of the N-terminal sequence of PPIase from porcine kidney

Cycle no.	Amino acid	Amount* (pmol)	Cycle no.	Amino acid	Amount* (pmol)	Cycle no.	Amino acid	Amount* (pmol)
1	Val	596	14	Glu	269	27	Lys	170
2	Asn	442	15	Pro	208	28	Val	118
3	Pro	554	16	Leu	180	29	Pro	96
4	Thr	119	17	Gly	245	30	Leu	146
5	Val	421	18	Arg	107	31	Thr	37
6	Phe	400	19	Val	132	32	Ala	111
7	Phe	429	20	Ser	44	33	Glu	68
8	Asp	403	21	Phe	142	34	Asn	84
9	Ile	345	22	Glu	90	35	Phe	133
10	Ala	313	23	Leu	103	36	Arg	55
11	Val	268	24	Phe	196	37	Ala	100
12	Asp	323	25	Ala	159	38	Leu	72
13	Gly	305	26	Asp	164			

PPIase was isolated from pig kidney cortex. After a 40–60% ammonium sulphate fractionation the protein was applied on a Sephadex cation exchange column at pH 8.5 and eluted with a linear KCl gradient (0–0.4 M). This was followed by gel filtration over Sephadex G-75 and chromatography on phenyl Sepharose or Fractogel TSK AF blue. The procedure resulted in a 780-fold purification and yielded material that migrated as a single 17 K band on denaturing polyacrylamide gels under reducing conditions. The N-terminal sequence was determined by automated Edman degradation using a 477A sequencer and an on-line 120A PTH analyser (both from Applied Biosystems). In addition to the 17 K PPIase, additional active protein species can be isolated which differ in M_r and isoelectric point. They will not be discussed further here.

* Yield of PTH amino acids at each step of the sequence analysis.

Fig. 1 Inhibition of PPIase by cyclosporin A. The activity of PPIase was determined by measuring the catalysis of the *cis* → *trans* interconversion of *cis*-Suc-Ala-Ala-Pro-Phe-4-nitroanilide. *a*, *Cis* → *trans* isomerization (●) in the absence of PPIase ($k = 7.4 \times 10^{-3} \text{ s}^{-1}$); (○) in the presence of 190 nM PPIase ($k > 1 \text{ s}^{-1}$); (Δ) in the presence of 190 nM PPIase + 7 μM CsA ($k = 9.0 \times 10^{-3} \text{ s}^{-1}$). The *cis* content of the substrate remained unaffected by the presence of CsA. *b*, Dependence of residual PPIase activity on CsA concentration. 3.1 nM PPIase were preincubated in the presence of varying concentrations of CsA and the remaining PPIase activity was determined. The substrate concentration was much smaller than K_M , therefore no deviation from first-order kinetics were observed in the presence of the inhibitor CsA. An inhibition constant $K_i = 2.6 \pm 0.4 \text{ nM}$ can be calculated from the data.

Methods. The *cis* → *trans* isomerization of the Ala-Pro peptide bond in the test peptide N-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide was measured in a coupled assay with chymotrypsin (Serva, Heidelberg), based on the ability of this protease to cleave the test peptide only when the Ala-Pro is *trans*^{1,17}. α-Chymotrypsin (21 μM) was preincubated with the appropriate concentrations of PPIase and CsA and the assay was started by manual mixing with 30 μl of the test peptide in the spectrometer cell at 10 °C (the final concentration of test peptide was 22 μM). The *trans* peptide was cleaved within the deadtime; hydrolysis of the 4-nitroanilide in the *cis* peptide is limited in rate by the *cis* → *trans* isomerization at Ala-Pro and was followed by the increase in absorbance at 390 nm.

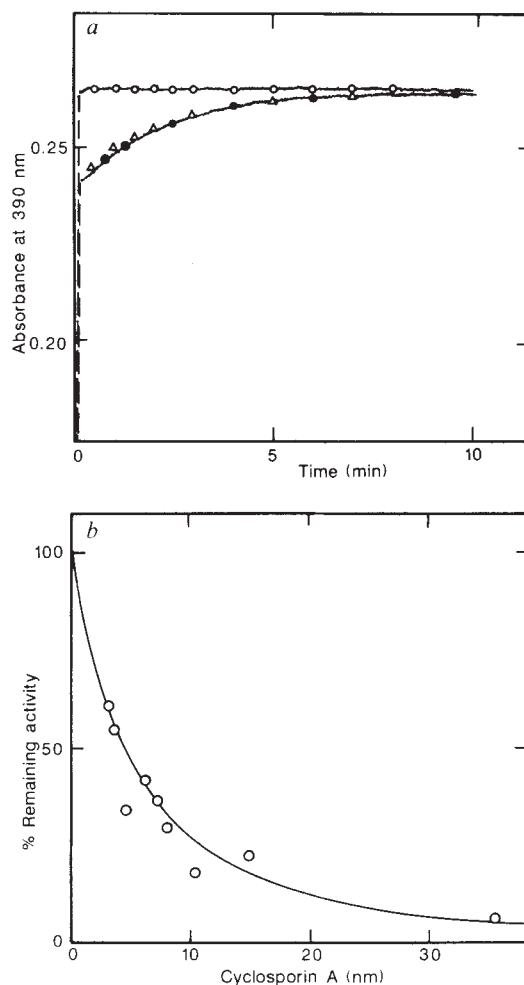
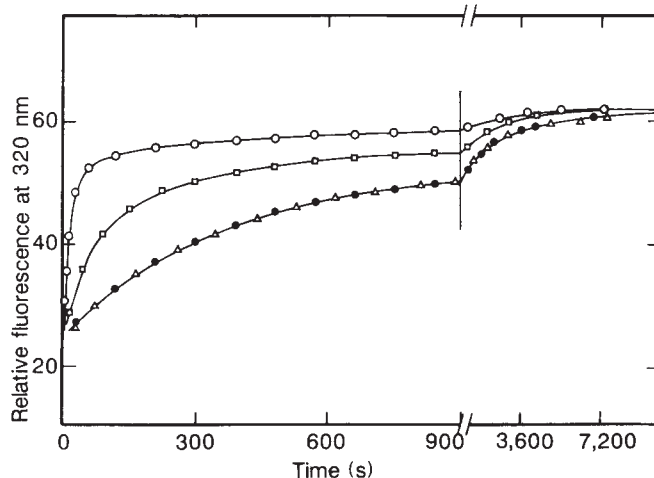


Fig. 2 Inhibition by CsA of the catalytic effect of PPIase on slow protein folding of RNase T1. The increase in fluorescence at 320 nm is shown as a function of the time of refolding in the absence of PPIase and CsA (●); in the presence of 0.35 μM PPIase (○); in the presence of 0.35 μM PPIase and 1.0 μM CsA (Δ); and in the presence of 0.35 μM PPIase and 0.5 μM CsA (□).

Methods. Recombinant RNase T1 was a gift from U. Hahn (Berlin). Unfolded protein was generated by a 2 h incubation of 13 μM RNase T1 in 8.0 M urea, 0.1 M Tris-HCl, pH 8.0 at 25 °C. Refolding was initiated by diluting 25 μl of the unfolded protein with 975 μl of 0.1 M Tris-HCl containing appropriate amounts of PPIase and CsA in the spectrophotometer cell at 10 °C. The final conditions for refolding were 0.33 μM RNase T1 in 0.2 M urea, 0.1 M Tris-HCl, pH 8.0 at 10 °C. Refolding was monitored by the increase in fluorescence at 320 nm (5 nm slit). Excitation was at 268 nm (5 nm slit). A Perkin-Elmer LS-5B fluorescence spectrophotometer was used.



slowly reacting cysteine residue of PPIase against modification with *p*HMB. Furthermore, completely SH-blocked PPIase no longer binds CsA, as could be shown in partition experiments of CsA between native PPIase and a large excess of the modified protein. The inactivation by *p*HMB (Fig. 3*b*) and the modification of the least accessible cysteine (Fig. 3*a*) show very similar kinetics, indicating that this cysteine is directly involved in the catalytic reaction of PPIase. In addition, binding of CsA inhibits the irreversible inactivation by *p*HMB (Fig. 3*b*). A kinetic deuterium isotope effect, observed when α-C deuterated Xaa amino acids in -Xaa-Pro- containing peptides are used as

substrates for PPIase¹⁴, suggests that the substrate is covalently attached to the enzyme in the course of catalysis, probably as a hemithioorthoamide, which would allow free rotation about the C-N bond.

We conclude from these data that cyclophilin and PPIase are closely related or identical proteins. Both are widely distributed in different organisms and cell types⁹; they are abundant (cyclophilin 0.1–0.4%, PPIase 0.4% of total protein^{7,15}), and they occur in different forms or isoenzymes (refs 6, 7 and unpublished observation by G. Fischer). The wide distribution of PPIase/cyclophilin and the highly conserved primary

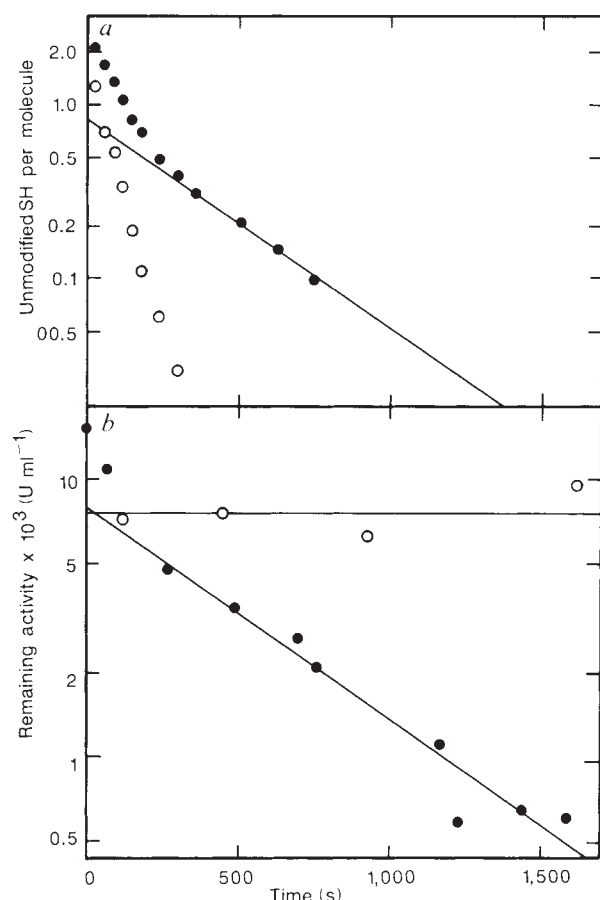


Fig. 3 Covalent modification of PPLase sulphydryls with *p*(hydroxymercuri)-benzoate in the absence (●) and in the presence (○) of 1.44 μM CsA. *a*, Time course of the modification of accessible thiols in 0.035 M HEPES buffer, pH 7.8 at 8.0 °C. PPLase (1.9 μM) was incubated with 28 μM *p*HMB and the extent of the modification reaction was followed by the increase in absorbance at 249 nm (with $\Delta\epsilon_{249} = 7,000 \text{ M}^{-1} \text{ cm}^{-1}$)¹⁸. A very fast modification reaction occurred within the deadtime of mixing, which reduced the concentration of *p*HMB to 16.1 μM. This burst may originate from the presence of rapidly reacting SH groups on the protein, but it probably results mainly from the presence of residual dithiothreitol in the enzyme solution. The slow part of the modification reaction was biphasic in the absence of CsA (●); 1.95 ± 0.2 SH groups per molecule reacted with $k_1 = 1.5 \times 10^{-2} \text{ s}^{-1}$ and 0.8 ± 0.3 SH groups per molecule reacted with $k_2 = 2.8 \times 10^{-3} \text{ s}^{-1}$. In the presence of CsA (○), 1.89 ± 0.2 SH groups per molecule were modified with a rate constant of $k = 1.55 \times 10^{-2} \text{ s}^{-1}$. The slow phase was absent. *b*, Loss of catalytic activity of PPLase (1.9 μM) in the course of the reaction with *p*HMB in the absence (●) ($k = 2.0 \times 10^{-3} \text{ s}^{-1}$) and in the presence (○) of 58.7 μM CsA. PPLase activity towards the test peptide was measured after a 1,200-fold dilution into the assay mixture. This high dilution was selected to decrease the concentrations of PPLase and of CsA below the K_i -value.

structure^{7,10,11} suggest a fundamental role in cellular metabolism. Both CsA-binding and prolyl isomerase activity involve binding to a peptide chain and this activity could, therefore mediate binding to polypeptide chains in the cell, for example in the course of, or after, protein biosynthesis. Such a role would, however, be at variance with the proposed role of CsA in the regulation of gene transcription¹⁶. More extensive conclusions about the cellular function of PPLase/cyclophilin are not warranted because at present it is not known whether it is the actual target of CsA during immunosuppression and whether it is important for slow steps in the *in vivo* protein folding process.

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Aminoacylation of RNA minihelices with alanine

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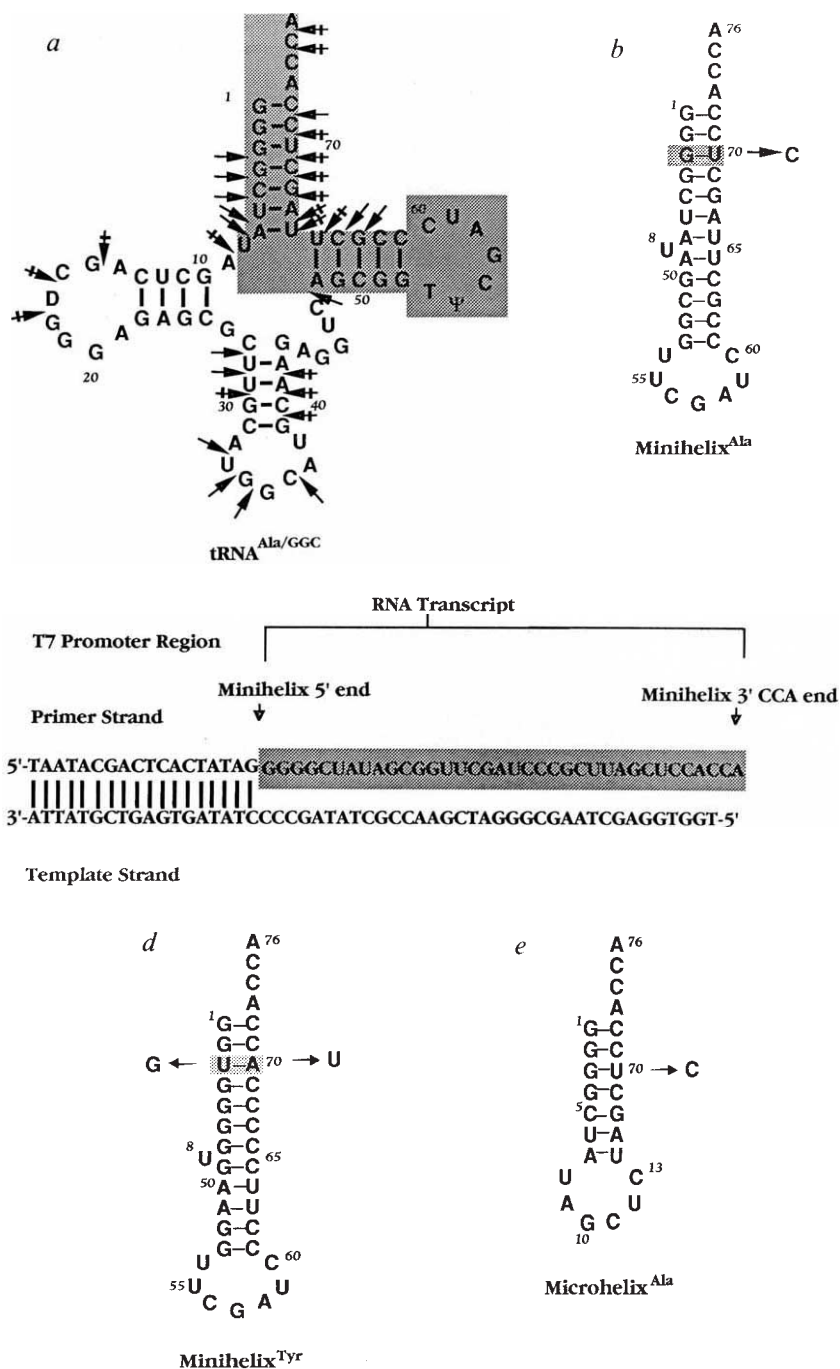
The genetic code is determined by both the specificity of the triplet anticodon of tRNAs for codons in mRNAs and the specificity with which tRNAs are charged with amino acids. The latter depends on interactions between tRNAs and their charging enzymes, and an advance in understanding such interactions was provided recently by the demonstration that a major determinant of the identity of alanine tRNA is located in the amino-acid acceptor helix. Multiple substitutions in many distinct parts of the molecule do not prevent aminoacylation with alanine¹. Substitution of the G3·U70 base pair with G3·C70 or A3·U70 in the acceptor helix prevents aminoacylation *in vivo* and *in vitro*, however¹, and the introduction of this base pair into tRNA^{Cys} (ref. 1) or tRNA^{Phe} (refs 1, 2) enables both to accept alanine. The importance of a single base pair in the acceptor helix and the results of recent footprinting experiments³ prompted us to investigate the possibility that a minihelix, composed only of the amino-acid acceptor-TΨC helix, could be a substrate for alanine tRNA synthetase. We show here that a synthetic hairpin minihelix can be enzymatically aminoacylated with alanine. Alanine incorporation requires a single G·U base pair, and occurs in helices that otherwise differ significantly in sequence. Aminoacylation can be achieved with only seven base pairs in the helix.

Figure 1*a* shows the sequence and cloverleaf structure of tRNA^{Ala/GGC}, with the amino-acid acceptor-TΨC helix and loop indicated by shading. In the three-dimensional structure of tRNA, the four arms of the cloverleaf are combined into two helical segments which are approximately at right angles, forming an L-shaped structure. The amino-acid acceptor and TΨC sequences form one continuous helix that terminates at the 3' end in four unpaired bases of the sequence XCCA_{OH}. In addition to the G3·U70 base pair, this structural motif of tRNA^{Ala} has intrachain base pairing and stacking interactions, which promote stability. A significant amount of the synthetase-tRNA interaction is concentrated in the acceptor-TΨC helix, as indicated by the large block of contiguous phosphodiester bonds that are protected from nuclease digestion by the bound synthetase (Fig. 1*a*).

The resulting hairpin minihelix^{Ala} (Fig. 1*b*) was synthesized using the template-primer system shown in Fig. 1*c*, and comprises twelve base pairs with a seven-nucleotide loop. The

Fig. 1 *a*, Sequence and cloverleaf structure of tRNA^{Ala}/GGC with nucleotides used in the construction of the minihelix indicated by shading. Phosphodiester bonds on tRNA^{Ala}/GGC that have been probed by nucleases in the presence and absence of bound alanine tRNA synthetase are also shown (arrows)³. Those which are protected from nuclease attack are shown by cross-hatched arrows. *b*, Synthetic hairpin minihelix based on the acceptor-TΨC helix of tRNA^{Ala}/GGC. A variant of this helix substituted C70 for U70. Numbering is based upon that for intact tRNA^{Ala}. *c*, Template-primer system for the synthesis of minihelices and microhelices. The sequence of minihelix^{Ala} is shown as an example. For the synthesis of other helices, a different DNA template strand was hybridized to the common 17-base pair T7 promoter strand. *d*, A synthetic minihelix based upon the acceptor-TΨC helix and loop of tRNA^{Tyr}. A second helix with a G3·U70 base pair was also made, as indicated. *e*, A synthetic microhelix based upon the acceptor helix of tRNA^{Ala}, with a loop comprised of nucleotides from U8 to C13.

Methods. The RNA minihelices and microhelices were synthesized in transcription reactions^{22,23} using T7 RNA polymerase and a synthetic DNA template featuring a double-stranded promoter region and a long 5' overhang corresponding to the transcribed sequence. T7 RNA polymerase was either purchased commercially (U.S. Biochemicals) or purified according to Grodberg and Dunn²⁴. Transcription reactions were incubated for four hours at 37 °C in a buffer containing 40 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, 1 mM spermidine, 50 µg ml⁻¹ bovine serum albumin, 80 mg ml⁻¹ polyethylene glycol (relative molecular mass, *M_r*, of 8,000), 20 mM MgCl₂, 4 mM of each nucleotide, and 2.5 U µl⁻¹ of T7 RNA polymerase. The DNA concentration in each strand was 0.25 µM. The reactions were terminated by phenol/chloroform extraction and then precipitated with ethanol. The full-length transcript was isolated by preparative gel electrophoresis from denaturing 20% polyacrylamide gels followed by electroelution or diffusion. The concentration of RNA in the preparations was determined by the absorbance at 260 nm at pH 7.5, 23 °C. A hyperchromicity value of 1.43 was used to correct the absorbance determinations. (This was obtained by measuring the absorbance of an RNA sample digested to completion with RNase T2 relative to an unhydrolysed sample.) For substrates that charged with alanine, the concentrations determined in this way (corrected when necessary for the minor fraction of molecules that terminated at their 3'-ends in C^{25,26}), agreed with the concentrations determined by calculating the alanine acceptance.



uridine at position 8 was included in the acceptor-TΨC structure because this nucleotide is conserved in all prokaryotic and cytoplasmic tRNAs⁴, is protected on one side by the bound enzyme (Fig. 1a), and is easily integrated into the acceptor-TΨC unit. We do not explore here whether this nucleotide is essential. Three additional minihelices were synthesized to test whether base substitutions in the acceptor stem exert the same effect on amino-acid acceptance that has been reported for the full-length tRNA^{Ala} (ref. 1). A derivative of minihelix^{Ala} was constructed in which U70 was replaced by C70 (Fig. 1b). This substitution has been shown to eliminate the aminoacylation function of tRNA^{Ala} (ref. 1). A minihelix that corresponds in sequence to that of the acceptor-TΨC helix of tRNA^{Tyr} was synthesized as well as a G3·U70 variant of that minihelix (Fig. 1d). The sequence of the acceptor-TΨC stem of tRNA^{Tyr} differs from that of tRNA^{Ala} at seven of 12 base pairs, providing a system in which to test whether G3·U70 retains its strong influence on

alanine identity in a different sequence context. In addition to these four minihelices, a fifth helix (the 'microhelix') was synthesized. This consisted of the seven base pairs of the acceptor helix of tRNA^{Ala}, connected by a six base-pair loop (Fig. 1e). In the sequence of microhelix^{Ala}, the sequence of the loop starts at U8 and continues into the 5' side of the D-stem, such that C13 is joined to U66. A G3·C70 variant of this sequence has also been constructed.

Aminoacylation of preparations of minihelix^{Ala} was examined at pH 7.5 and 37 °C. As a negative control, wild-type tRNA^{Tyr} was used as a substrate. At a concentration of 4.5 µM, the minihelix is aminoacylated at catalytic concentrations (15 nM) of alanine tRNA synthetase, whereas there is no detectable aminoacylation of the control under the same conditions (Fig. 2a). At higher concentrations of the enzyme (300 nM), near-complete aminoacylation of the minihelix is observed within 20 min (Fig. 2a, inset).

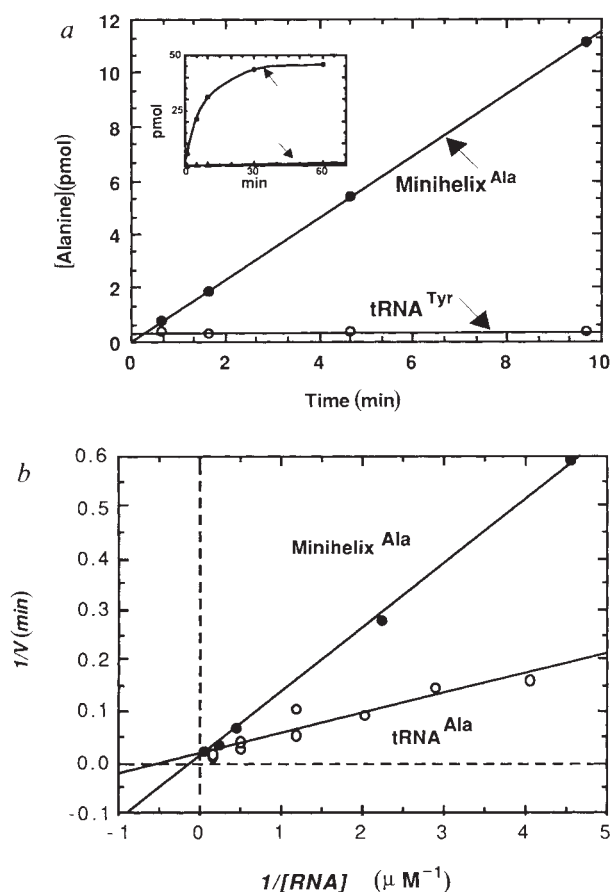


Fig. 2 *a*, Aminoacylation of tRNA^{Tyr} and of $\text{minihelix}^{\text{Ala}}$. The assays were carried out at pH 7.5, 37 °C, as previously described^{20,21} in a buffer containing 50 mM sodium phosphate (pH 7.5), 100 $\mu\text{g ml}^{-1}$ bovine serum albumin, 20 mM potassium chloride, 10 mM MgCl_2 , 20 mM β -mercaptoethanol, 4 mM ATP, 20 μM alanine, 2.4 μM [2,3- ^3H] alanine and 4.5 μM $\text{minihelix}^{\text{Ala}}$ RNA or 5 μM tRNA^{Tyr} . The reactions were initiated by adding purified alanine tRNA synthetase to a final concentration of 15 nM. The inset shows an experiment that compares the aminoacylation of 2.5 μM $\text{minihelix}^{\text{Ala}}$ with 5 μM tRNA^{Tyr} at an alanine tRNA synthetase concentration of 300 nM. *b*, Plot of inverse velocity against inverse RNA concentration for the aminoacylation of tRNA^{Ala} and $\text{minihelix}^{\text{Ala}}$. Each point was obtained from the determination of the initial linear rate of aminoacylation at varying substrate concentrations of either the minihelix (over a ten-min interval) or of tRNA^{Ala} (over a 2.5-min interval). The concentration of purified alanine-tRNA synthetase was 15 nM. The reaction conditions were otherwise similar to those in Fig. 2*a*.

A determination of the initial rates of aminoacylation at several substrate concentrations allowed the individual kinetic parameters, the Michaelis constant K_m , and k_{cat} to be determined separately for two substrates. Figure 2*b* shows a plot of inverse velocity against inverse RNA concentration. The plots for both tRNA^{Ala} and $\text{minihelix}^{\text{Ala}}$ extrapolate to nearly the same intercept on the ordinate, indicating that the enzyme has a similar maximal velocity for the two substrates. The K_m parameter is about fivefold higher for the minihelix, however, suggesting that it binds less efficiently to the enzyme (Table 1). This factor of five corresponds to about one kcal mol^{-1} , which is sufficiently small to be accounted for by one or two van der Waals' contacts that may be absent in the enzyme-minihelix complex, as compared to the analogous complex with wild-type tRNA^{Ala} . Because k_{cat} and K_m are close to values observed for the native tRNA, the fragment must possess the structures

Table 1 Kinetic parameters for the aminoacylation of tRNA^{Ala} and of helical fragments with *Escherichia coli* alanine tRNA synthetase

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
tRNA^{Ala}	2.0	0.89	4.4×10^5
$\text{Minihelix}^{\text{Ala}}$	11.4	1.5	1.3×10^5
G3·U70 $\text{minihelix}^{\text{Tyr}}$	8.8	0.48	5.3×10^4
$\text{Microhelix}^{\text{Ala}}$	35.9	0.28	7.7×10^3

Aminoacylation reactions were carried out as described in the Fig. 2 legend. The concentration ranges of each of the substrates used to measure the kinetic parameters were as follows: tRNA^{Ala} , 0.25–6.4 μM ; $\text{minihelix}^{\text{Ala}}$ and G3·U70 $\text{minihelix}^{\text{Tyr}}$, 0.22–25 μM ; and $\text{microhelix}^{\text{Ala}}$, 0.5–25 μM . Purified alanine tRNA synthetase was used at a concentration of 15 nM (as determined by active site titration^{19,20}) for all kinetic assays. The parameters for all of the RNA substrates were determined at a saturating ATP concentration (4 mM) but at a subsaturating concentration (20 μM) of alanine. The high K_m of alanine tRNA synthetase for alanine (0.25 μM ²⁰) required prohibitively high radioactive alanine concentrations to achieve saturation. In earlier work, it has been shown that the K_m for tRNA^{Ala} shows little sensitivity to the concentration of alanine²¹.

required for both binding and passage through the transition state.

The G3·C70 variant of $\text{minihelix}^{\text{Ala}}$ was synthesized and found to be completely inactive for aminoacylation with alanine, even under conditions where a relatively high (20-fold higher than that used for the kinetic parameter determination) enzyme to substrate ratio was used (Fig. 3*a*). This suggests that the G3·U70 base pair is also important in determining the amino-acid identity of the minihelix, as it does in the intact tRNA^{Ala} (ref. 1).

A 'tyrosine' minihelix was also synthesized (Fig. 1*d*) and tested for aminoacylation with alanine. As in the case of $\text{minihelix}^{\text{Ala}}$, the presence of G3·U70 is required for efficient aminoacylation. The rate of aminoacylation of the G3·U70 variant of $\text{minihelix}^{\text{Tyr}}$ is only 2.5-fold slower than that of the $\text{minihelix}^{\text{Ala}}$. This result indicates that alanine tRNA synthetase does not exert significant catalytic discrimination against the six base pairs that are not common to $\text{minihelix}^{\text{Ala}}$ and G3·U70 $\text{minihelix}^{\text{Tyr}}$. On the basis of a determination of the kinetic parameters for G3·U70 $\text{minihelix}^{\text{Tyr}}$, the rate reduction can be largely attributed to the k_{cat} parameter (Table 1).

The amino-acid acceptor portion of tRNA^{Ala} comprises only seven base pairs. We observed that a 'microhelix' based on just the sequence of the tRNA^{Ala} acceptor stem can be completely aminoacylated by the enzyme, at a reduced rate relative to that of $\text{minihelix}^{\text{Ala}}$ (Fig. 3*b*). The kinetic parameters for $\text{microhelix}^{\text{Ala}}$ were determined under the same conditions as for the minihelices (Table 1). The K_m for $\text{microhelix}^{\text{Ala}}$ is 18-fold higher and the k_{cat} is threefold lower than the respective parameters for tRNA^{Ala} . The differences between the values for $\text{microhelix}^{\text{Ala}}$ and $\text{minihelix}^{\text{Ala}}$ demonstrate the effect of the removal of sequences corresponding to the T ψ C stem and loop. The greater effect on K_m suggests that, in the progressive deletion of sequences outside the acceptor stem, some contacts with the enzyme have been lost. On the basis of the relative Michaelis constants (K_m) for tRNA^{Ala} and $\text{microhelix}^{\text{Ala}}$, the decrease in binding energy is about 1.7 kcal mol^{-1} . The lost contacts do not seem to be crucial to catalysis however, because the effect on k_{cat} is relatively small. Other experiments showed that efficient aminoacylation of the microhelix requires the G3·U70 base pair (data not shown).

Although the influence of the G3·U70 base pair on the K_m parameter is important in discriminating tRNA^{Ala} from other tRNAs, there is evidence that the k_{cat} parameter is more important². For certain tRNAs, some or all of the sequences which determine identity are located in the anticodon^{6–10}. The data in Table 1 show that for $\text{microhelix}^{\text{Ala}}$, the k_{cat} parameter is within a factor of three of that for wild-type tRNA^{Ala} . Thus, once bound

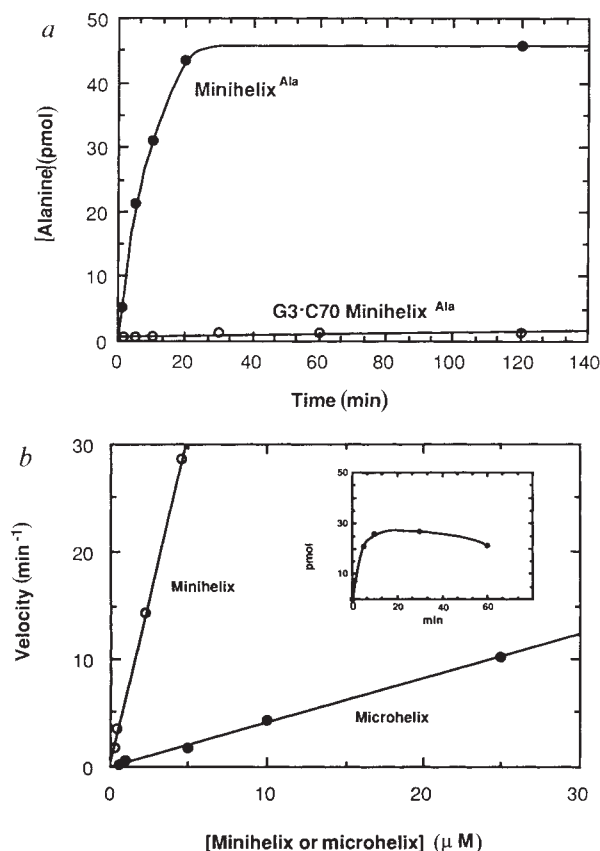


Fig. 3 *a*, Initial rate of aminoacylation with alanine of the 'wild-type' and G3·C70 variant of minihelix^{Ala}. The RNA substrates were present at a concentration of 5 μM, and alanine tRNA synthetase was added to a final concentration of 300 nM, which is a twentyfold increase over the concentrations used in the determination of kinetic parameters. *b*, Initial velocity of aminoacylation with alanine against the concentration of mini- and microhelix^{Ala}. The concentrations of the RNA substrates were as follows: minihelix^{Ala}, 0.25–4.5 μM; microhelix^{Ala}, 0.5–25 μM. Alanine tRNA synthetase was added to a final concentration of 15 nM, and the reaction conditions were similar to those in Fig. 2. The inset shows an aminoacylation experiment where the concentration of microhelix^{Ala} was 3 μM and the concentration of alanine tRNA synthetase was 1 μM.

to the enzyme, the microhelix is aminoacylated at a comparable rate to that of intact, native tRNA^{Ala}.

Although we have confirmed that G3·U70 acts as a positive determinant for recognition by alanine tRNA synthetase, we cannot rule out the possibility that tRNA^{Ala} possesses negative determinants that reduce its interactions with non-cognate synthetases. These negative determinants may, in part, lie outside the acceptor-TΨC helix.

Earlier studies suggested that partial tRNA molecules or tRNA fragment combinations might be substrates for aminoacylation *in vitro*, although the overall yields of aminoacylation were 5% or less in some cases^{11,12}. There are examples of naturally-occurring tRNAs that are truncated versions of the typical four-armed cloverleaf pattern, although they are not as reduced in size as the molecules studied here. In the sequence analyses of several mammalian mitochondrial genomes, a tRNA^{Ser/AGY} has been identified that has an ordered structure in which the D-stem and loop are absent^{13,14}. Also, at least one other enzyme involved in tRNA metabolism (RNase P) is able to use RNA substrates that are smaller than a complete tRNA¹⁵.

It has been speculated that, during an early stage of evolution, small tRNA molecules were esterified with amino acids in a system that was a precursor to protein synthesis (see summary

in ref. 16). These molecules could have had sequences that influenced the selection of the amino acid attached by the primordial synthetase¹⁷. The data presented here show that, for one amino acid at least, information sufficient for its specific attachment to an RNA molecule can be encoded by sequences proximal to the 3'-amino-acid attachment site.

It has been speculated that primitive RNA genome molecules were tagged by tRNA-like structures in early evolution¹⁸. These tags might have been used by a primordial enzyme as replication initiation points. In the light of these suggestions, our results invite speculation that these tags could have been short RNA minihelices, which after serving as primitive RNA telomeres, were released to become pre-tRNAs upon which the first peptides were synthesized.

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Long-range structural changes in proteinase K triggered by calcium ion removal

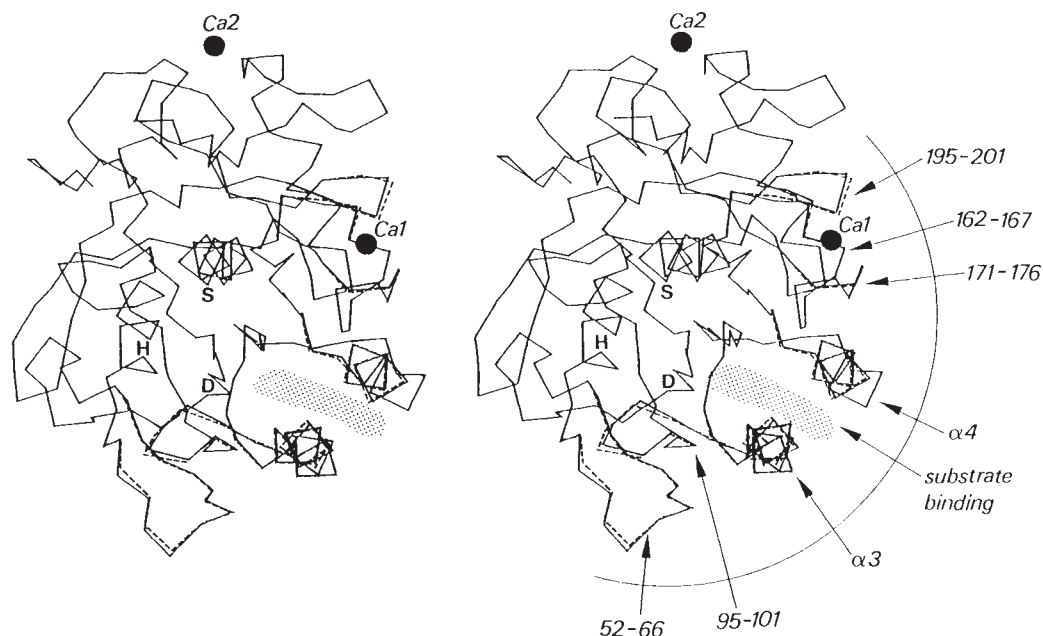
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The X-ray crystal structure of the subtilisin-type enzyme proteinase K at 1.5 Å resolution¹ shows that it has two binding sites for Ca²⁺. Scatchard analysis² indicates that one Ca²⁺ binds tightly, with $pK_{0.5} = 7.6 \times 10^{-8} M^{-1}$, and the other only weakly. Although Ca²⁺ is not directly involved in the catalytic mechanism and is 16.6 Å away from the α-carbon atoms of the catalytic triad Asp 39–His 69–Ser 224, the activity of proteinase K towards the synthetic substrate succinyl-Ala-Ala-Ala-p-nitroanilide drops slowly to ~20% of its original value when it is depleted of Ca²⁺. This is not due to autolysis of the enzyme². The X-ray crystal structure of Ca²⁺-free proteinase K shows that removal of Ca²⁺ from the tight binding site triggers a concerted domino-like movement of five peripheral loops and of two α-helices. At a distance of 25 Å from this

Fig. 1 Stereo view of Ca^{2+} -superposition of Ca^{2+} -containing and Ca^{2+} -free proteinase K molecules. The least-squares fit procedure for the whole molecule gives an r.m.s. deviation of 0.22 Å. If only the 'isomorphous' atoms that are in identical positions within ~ 0.1 Å are included, the r.m.s. deviation is 0.04 Å. Sections where differences in atomic coordinates exceed 0.2 Å have an r.m.s. deviation of 0.74 Å and are shown in broken lines for Ca^{2+} -free proteinase K. The Ca^{2+} -binding sites Ca1 and Ca2, the substrate-binding site and the segments that move after removal of Ca^{2+} are indicated. Significant autolysis of Ca^{2+} -free proteinase K occurs only 48 h after incubation¹; crystals were obtained after 12 to 24 h.

Methods. Proteinase K (Merck, Darmstadt) was dissolved to 10% concentration in 50 mM Tris-HCl, pH 7.8, 280 mM Na-EDTA. Vapour diffusion against 0.8 M NaNO_3 yielded crystals isomorphous with the native Ca^{2+} -containing enzyme, of space group $P4_32_12$, $a = b = 68.3$ Å, $c = 108.4$ Å. Film data to 2.18 Å resolution were collected on an Arndt-Wonacott camera (Elliott GX20 generator, CuK_α graphite monochromator); there were 11,634 independent reflections ($R_{\text{merge, sym}, 1} = 5.2\%$) with 85% $> 1\sigma_F$. Phasing was with a 1.5 Å 'native' model¹; manual model building was based on $2F_o - F_c$ and 'omit' maps (FRODO¹²; Evans and Sutherland, PS 300), followed by restrained least squares refinement cycles¹³. R -factor is 18.1% for all data $> 3\sigma_F$; model consists of C, N, O and S atoms and 94 water oxygens with $B < 30$ Å. Drawn with the program in ref. 14.



calcium-binding site, the geometry of both the secondary substrate binding site and of the catalytic triad is affected by this movement thereby reducing the activity of the enzyme.

The enzyme activity of proteinase K from the mould *Tritirachium album* Limber is so high that it will even digest keratin^{3,4}; it is a calcium-dependent enzyme. The catalytic triad Asp 39-His 69-Ser 224 and the two strands of the substrate-binding region corresponding to residues 100-102 and 132-135, form an antiparallel triple β -sheet structure with the substrate, and have been characterized by crystal structure analysis of proteinase K bound to two different peptide chloromethane inhibitors^{5,6}. The more strongly binding of the two Ca^{2+} -binding sites (Ca1) is well defined and coordinates in the form of a pentagonal bipyramid: if the two side-chain carboxyl oxygen atoms of Asp 200 are taken as one ligand, then the Pro 175 C=O and Asp 200 $\text{O}_{81}\text{O}_{82}$ are in apical positions, with Val 177 C=O and four water molecules occupying the equator. In contrast, the coordination sphere of the weaker binding site (Ca2) is incompletely defined in the X-ray structure, by Thr 16 C=O, Asp 260 $\text{O}_{81}\text{O}_{82}$ and two water molecules¹.

If these Ca^{2+} ions are removed using EDTA and subsequent gel filtration, the enzyme activity of proteinase K decreases by $\sim 80\%$, falling with $t_{1/2} \sim 3$ h. Activity can be restored to only 28% by excess Ca^{2+} (ref. 2). This behaviour is not associated with autolysis of the enzyme, which becomes significant only after 48 h incubation², so we interpret it as resulting from structural changes induced when proteinase K is depleted of Ca^{2+} . We therefore undertook an X-ray crystallographic study of the Ca^{2+} -free enzyme; methods are given in the legend to Fig. 1.

Figure 1 shows that there are no cuts in the polypeptide chain and that the main portion of the molecular structure of proteinase K, including the weaker binding site Ca2, is only marginally affected by removal of Ca^{2+} if at all. Significant structural changes giving shifts greater than 0.2 Å in atomic coordinates occur in a coherent peripheral section. These are indicative of a concerted motion triggered by the substitution of $\text{Ca}^{2+} \cdot 4\text{H}_2\text{O}$ by two water molecules at the Ca1 binding site (Fig. 2); this

motion propagates in a domino-like way through adjacent segments of the molecule. These segments involve the two peripheral α -helices, $\alpha 3$ and $\alpha 4$, and the five loops comprising residues 195-201, 162-167, 171-176, 95-101 and 52-66, which make contact through van der Waals interactions, hydrogen bonds and a salt bridge (Table 1). These subtle local changes in molecular structure are associated with more severe positional shifts, deletions and additions of water molecules in the first hydration sphere, suggesting that alterations in the peripheral tertiary hydrogen bonding are substantial. The motion is bounded in an anticlockwise sense (Fig. 1) by a loose connection between loop 195-201 and the protein carboxyl terminus, which acts as buffer, and in 'clockwise sense' by loop 52-66 which is 30 Å away from Ca1 and is exposed to solvent. The movement of the tyrosine residues in loop 52-66 was also detected by circular dichroism², suggesting that these structural changes that occur on removal of Ca^{2+} are an intrinsic property of the molecule, and not a result of crystal lattice effects.

The secondary substrate binding site is formed by two strands, from residues 100-102 and 132-135. The latter is linked with site Ca1 through the loop of amino acids 171-176. The shift expected after Ca^{2+} removal (accompanied by isomerization of *cis* Pro 171 to the *trans* form) with concomitant reduction in enzymatic activity², does not occur; probably because loop 171-176 is tightly anchored on to helix $\alpha 6$ by a disulphide bond from Cys 178-Cys 249 (Fig. 2a and b). Instead, the peptide carbonyl group of Gly 100, which is part of loop 95-101 and 25 Å away from Ca1, is shifted by ~ 0.8 Å, so that hydrogen bonding to the substrate is diminished or even impeded (Fig. 2c). Side chains of catalytic triad sequence Asp 39-His 69-Ser 224 are rotated somewhat. Consequently, the hydrogen bond between Ser 224 O_γ and $\text{N}_\epsilon 2$ His 69, which is necessary for the catalytic proton transfer, is lengthened from 3.01 Å in the 'native' Ca^{2+} -bound enzyme to 3.54 Å in the Ca^{2+} -free state (Fig. 2d).

The shifts in atomic positions at the catalytic centre can explain the reduction in enzymatic activity of proteinase K on removal of Ca^{2+} . That the activity is not lost completely is

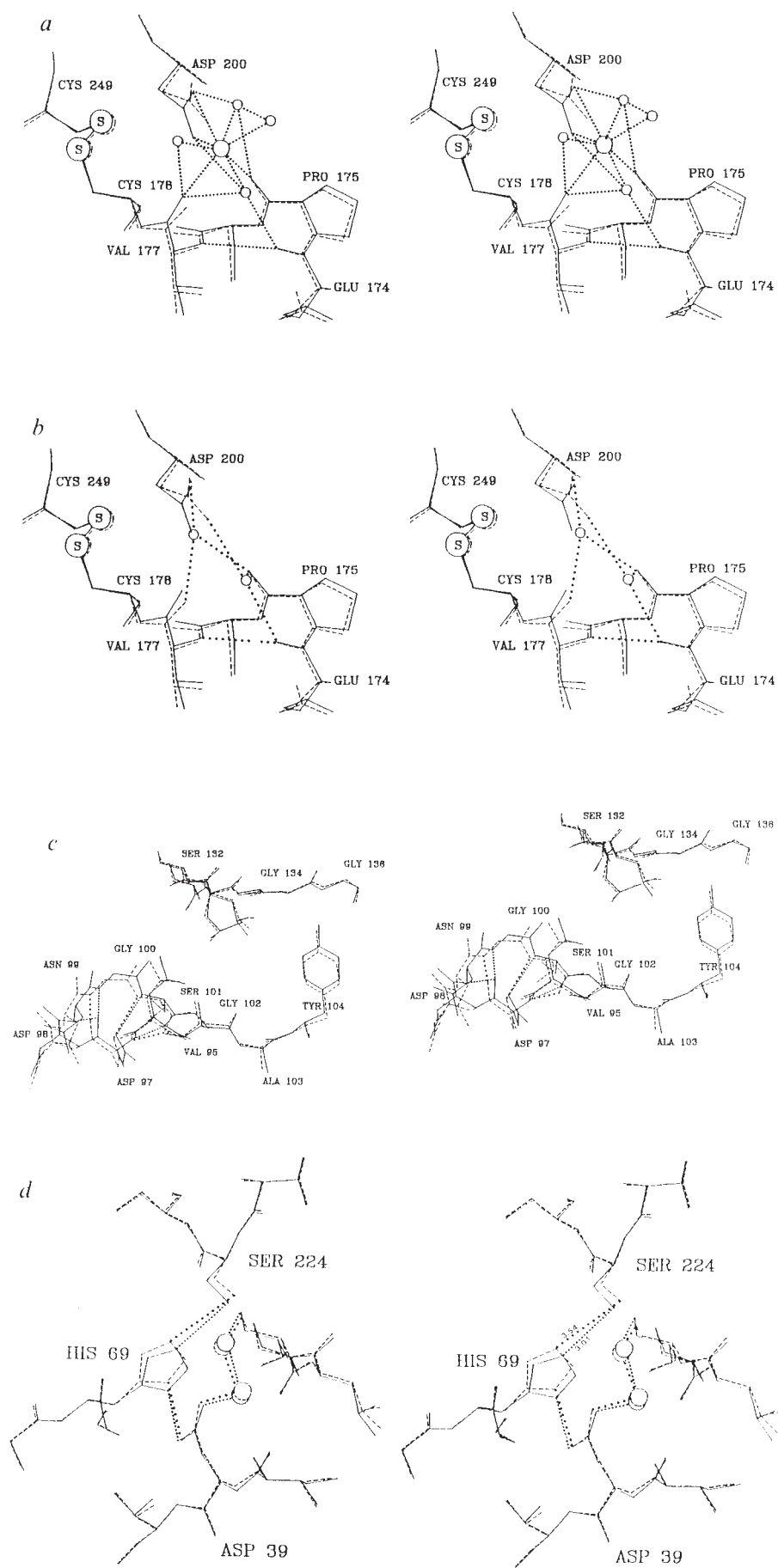


Fig. 2 *a-d*, Stereo views of different sections of the molecular structure of 'native' proteinase K containing Ca^{2+} (solid lines) and of Ca^{2+} -free proteinase K (broken lines). Hydrogen bonds and Ca^{2+} coordination bonds are indicated by dotted lines, the smaller dots referring to Ca^{2+} -containing proteinase K and larger dots to Ca^{2+} -free proteinase K. Drawn with the program from ref. 14. *a, b*, The Ca^{2+} -binding site Cal drawn with (*a*) and without (*b*) Ca^{2+} . Coordination and hydrogen bonds indicated by dotted lines, small spheres are water molecules, the larger sphere is Ca^{2+} . The disulphide bond is marked S; Cys 249 is attached to helix $\alpha 6$. The secondary substrate-binding site (segment 130-134) that does not shift significantly when Ca^{2+} is removed is at the lower left (not shown). *c*, The secondary substrate-binding site. The structure of segment 132-135 is almost unaffected by removal of Ca^{2+} , whereas the segment 100-102 changes position and conformation. The hydrogen bonds with Asp 97 (dotted lines) are all lengthened in the Ca^{2+} -free enzyme by 0.3-0.5 Å. *d*, View of the catalytic site. Main-chain atoms are almost unaffected, but side chains shift so that the length of the critical hydrogen bond between Ser 224 O_γ and $\text{N}_{\epsilon 2}$ His 69 increases by 0.53 Å when Ca^{2+} is removed. Water molecules are drawn as spheres.

Table 1 Intramolecular interactions between segments which move domino-like when Ca^{2+} is removed from proteinase K

Segment*	Interactions
195-201	{ Ala 164 C=O...Tyr 195 N Ala 166 N...Tyr 196 C=O
162-167	{ Asp 165 O_δ ...W326...Ser 197 O_γ Val 177 C=O Glu 174 C=O
171-176	{ Asp 200 C { $\text{O}_{\delta 1}$...W283 $\ominus$ $\text{O}_{\delta 2}$...W282...Pro 175 C=O
142-154 ($\alpha 4$)	{ Asn 142 $\text{N}_{\delta 1}$...Ser 173 C=O Ala 146 C=O...W317...Ser 176 C=O
95-101 and $\alpha 3$	{ Asp 112 C { $\text{O}_{\delta 1}$... $\text{N}_{\eta 1}$ $\ominus$ $\oplus$ C Arg 147 $\text{O}_{\delta 2}$... $\text{N}_{\eta 2}$
52-66	{ Met 111 $\equiv$ (Ala 144, Leu 148) (Trp 91, Val 95, Leu 96) $\equiv$ (Tyr 59, Tyr 60, Tyr 61) Asp 98 C=O... $\text{N}_{\delta 2}$ C { $\text{O}_{\delta 1}$...His 69 N Asn 67 (active site)

Dashed lines represent hydrogen bonds $\text{X}-\text{H}\cdots\text{A}$, with $2.68 \text{ \AA} \leq \text{X}\cdots\text{A} \leq 3.20 \text{ \AA}$; triple lines represent hydrophobic interactions. The two peripheral α -helices involved in the movement are $\alpha 3$ (residues 103 to 121) and $\alpha 4$ (residues 142 to 154).

*See Fig. 1.

probably due to the relatively subtle nature of the structural changes. The slow rate of this reduction in activity can be explained by the tightly bound Ca^{2+} at site Ca1 resisting chelation by EDTA, together with the associated movements in the hydration sphere which probably dampen this process even further. The altered hydration of Ca^{2+} -free proteinase K could trap the enzyme in a new energy minimum (calculations to be published). This would explain why the activity of proteinase K is not fully recovered after addition of excess Ca^{2+} (ref. 2).

Proteinase K does not autolyse after removal of Ca^{2+} for 48 hours (ref. 2), probably because the disulphide bridge Cys 178-Cys 249 stabilizes the geometry of the 'empty' Ca1 binding site, thereby preventing even partial unfolding. Two related subtilisins, BPN and Novo, also have two Ca^{2+} binding sites, but in different positions⁷⁻¹¹, and they do not contain disulphide bridges. This could explain why they unfold after depletion of Ca^{2+} and become sensitive to enzyme attack to autolyse readily.

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Advances in two-dimensional electrophoresis

S.M. Hanash and J.R. Strahler

New developments in the first dimension step of two-dimensional electrophoresis have expanded the utility of the technique in cell and molecular biology.

WHILE two-dimensional electrophoresis is unsurpassed by any other technique for simultaneously resolving hundreds of polypeptides, its potential to contribute to our understanding of molecular processes remains largely untapped. Because of the difficulty in achieving adequate reproducibility and truly high resolution, the technique has successfully been practised in a relatively small number of laboratories. Those difficulties notwithstanding, it was evident at a two-dimensional electrophoresis meeting held in Vienna last November* that the technique has been used to identify a good number of novel proteins that play a role in cell activation, proliferation, differentiation and other functions¹. Genes corresponding to proteins detected on two-dimensional gels have been cloned by several groups, including our own.

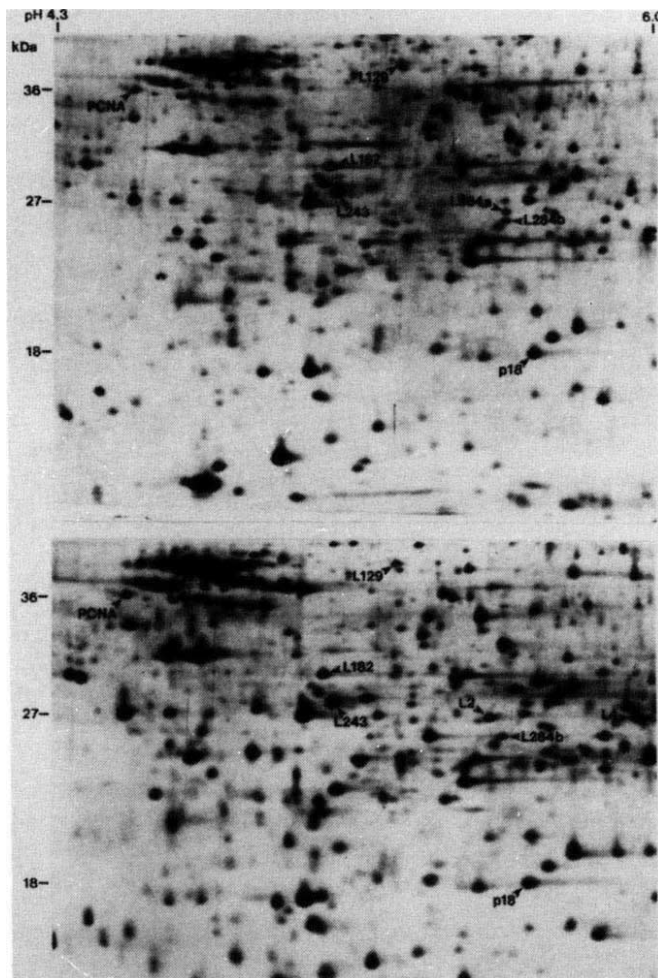
Immobilized pH gradients

A major contributor to the variability seen in two-dimensional gel patterns within and between laboratories is the carrier ampholyte (CA) which is the determinant of the first dimension separation, based on charge. Detailed comparisons of patterns generated for the same cell material in separate laboratories have been difficult, hampering efforts to establish collective protein databases of two-dimensional gel information. Problems with CA-based separations include: gaps in the gel patterns, resulting from discontinuities in the pH gradient, which varies between sources of CA; drift in the focusing pattern during electrophoresis (particularly severe for basic polypeptides); and difficulty in producing pre-cast gels suitable for two-dimensional electrophoresis.

These difficulties prompted us to develop a two-dimensional approach that utilizes immobilized pH gradients (IPG) for the first dimension separation^{2,3}. With IPG, the pH gradient is an integral part of the polyacrylamide gel matrix⁴. The technique overcomes many of the problems encountered when using carrier ampholytes. The pH separation range can be defined from broad to extremely narrow, basic polypeptides can be resolved well, and precast gels can be used. IPG gels can also be loaded with preparative amounts of protein suitable for polypeptide sequencing, because they have higher loading capacities than CA gels.

In the IPG method, gradient slab gels of

Fig. 1 Comparison of the polypeptide patterns of two separate Epstein-Barr virus-transformed lymphoblastoid cell lines, generated with two-dimensional gel electrophoresis using immobilized pH gradient gels prepared from separate castings. Arrows point to polypeptides of interest to the study of acute leukaemia, some of which have been sequenced and their corresponding genes cloned^{6,7}. Some of the differences between the protein patterns are due to genetic polymorphisms, and others to stage of differentiation^{7,8}.



0.5 mm in thickness are first cast on a GelBond PAG film in a large format (24 cm wide with a 17-cm pH gradient separation distance). For most applications, the gradient spans three pH units: 4-7 for acidic and neutral polypeptides, and 6.5-9.5 for neutral and basic molecules. Each slab gel yields roughly seventy 3.5-mm-wide first dimension strips. Following extensive washing with deionized water, the gels are dried and wrapped in thin plastic film, and can be stored at -20 °C for several months.

Prior to the second dimension electro-

phoresis step, the desired number of strips are cut and rehydrated, as a group, with a solution of urea, non-ionic detergent, dithioerythritol and dilute acetic acid. The strips are then placed on the cooling plate of a humidified, sealed electrofocusing apparatus, and sample applicators of between 40 and 80 µl in capacity are placed directly onto the surface of the gel.

The degree of sample entry into the gel is critical. We have found that the best results are obtained using dilute samples with a low salt concentration to which carrier ampholytes have been added, with

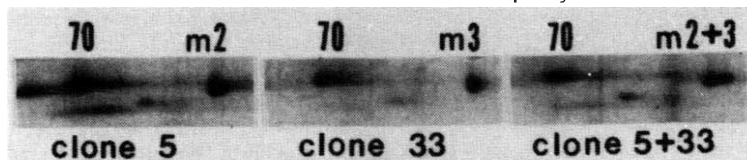


Fig. 2 Close-up sections of two-dimensional gels prepared with narrow immobilized pH gradients (pH 6.3-6.8) in the first dimension. Two mutants of polypeptide 70 prepared from separate mutagenized lymphoblastoid cell lines phenotypically identical in broad-range CA-based gels are clearly resolved by immobilized pH gradients. The mutants differ in charge by <0.003 pH units.

* The International 2-Dimensional Electrophoresis Meeting, Vienna, Austria, 8-11 November 1988.

a low voltage gradient across the gel (30 V cm^{-1}) for the first hour. The voltage can then be increased to 5,000 V (0.1 mA per strip, maximum) for 22–24 hours.

The second dimension electrophoresis step can be performed using vertical or horizontal slab gels. Figure 1 illustrates the reproducibility and resolution which can be achieved using relatively broad pH gradients. An example of the resolution which can be achieved using narrow gradients is shown in Fig. 2.

Other developments

It should be emphasized that in practice, no one technique can lead to the simultaneous visualization of all cellular polypeptides. Thus, for some applications, the selective analysis of protein subsets of interest (such as the low-abundance soluble nuclear proteins) by prior cell fractionation is advantageous.

Additional approaches include the use of separation modes other than isoelectric focusing for the first dimension separation. The direct coupling of microbore HPLC to SDS electrophoresis is particularly promising. In such a system, various HPLC separation modes, from reversed phase to ion exchange, could be used to perform the first dimension separation. In a previous study of plasma proteins, we observed that the resolving power of ion exchange HPLC is equivalent to isoelectric focusing with CA, based on the separation of transferrin variants⁵.

Another promising development is the use of capillary electrophoresis as the first dimension separation mode. An effort is also being made to overcome some of the problems experienced with CA-based separations to eliminate gaps in the gels and to allow the production of precast focusing gels containing urea which can be stored for lengths of time.

It is becoming clear that, after an initial lag behind DNA technology, most of the practical knowledge is now in place to allow a major leap forward in our understanding of the organization, structure and function of the principal cellular components which mediate cell function, namely, the proteins. □

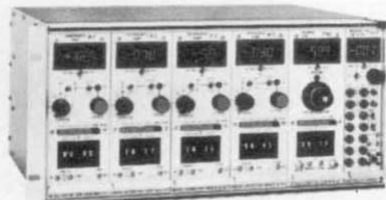
Sam M. Hanash and John R. Strahler are at the University of Michigan Medical Center, R4451 Kresge I, Box 0510, Ann Arbor, Michigan 48109-0510, USA. For more information, fill in reader service number 100.

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Of peptides and DNA

Neurobiology and neurochemistry will be the talk of the town in Miami next week during the Miami Bio/Technology Winter Symposium. Besides an array of patch-clamps and recorders, the exhibits will feature products for studying peptides, and the nucleotides which code for them.

MEDICAL Systems Corporation has a new **micropump system** for controlling the ejection of fluids from micropipettes in neurobiotechnological studies (*Reader Service No. 101*). The company's Neuro-



Medical Systems' micropump for neurobiology. Phore BH-2 has both a pneumatic and an ionophoretic pump module, and can deliver intracellular or extracellular ejections of minute volumes using up to five pumps in parallel. The ejection schedule for each pump can be programmed independently. Medical Systems Corporation also offers multibarrel micropipettes for use with the NeuroPhore BH-2 system to enable researchers to eject several different fluid combinations for investigating cell responses. The company is exhibiting in booth 78.

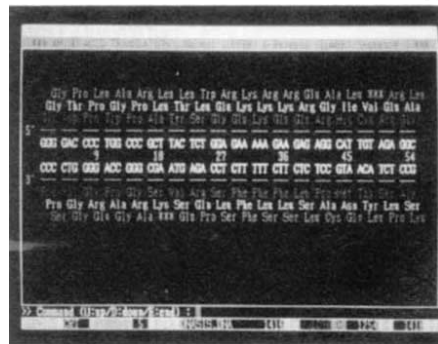
United States Biochemical, exhibiting in booths 62 and 63, offers a range of **neuropeptides** which affect the central nervous system (*Reader Service No. 102*). The company is noteworthy for producing Bombesin and MBHA-peptide resin, the preferred resin for producing alpha-carboxamide peptides, but they also sell endorphins, delta sleep-inducing peptide, alpha-melanocyte stimulating hormone, and enkephalins. Purified amino acids and derivatives for peptide synthesis can also be chosen from USB's product listing, which contains over 6,000 products. The company has recently been appointed by Perkin-Elmer Cetus as a co-distributor of Perkin-Elmer Cetus's GeneAmp reagents for performing the polymerase chain reaction.

Software smarts

Beckman has updated its popular MicroGenie **sequence analysis software** to include the capability to calculate circular restriction maps, including double and multiple digests (*Reader Service No. 103*). The new feature allows the user to enter and store lists of restriction enzymes and their recognition sites, even before analysis, and to calculate restriction fragment sizes based on gel mobilities. The new version retains the old MicroGenie's

shotgun DNA sequencing function, where sequences can be merged and scanned for overlaps, insertions, deletions and mismatches, and the homology search and alignment function, which draws upon the GenBank nucleic acid database and the National Biomedical Research Foundation protein sequence database. With MicroGenie, it is also possible to predict protein secondary structures and probabilities, determine residue and codon frequency, predict and plot protein hydrophobicity, and find inverted repeat regions and calculate the free energy. Beckman offers a free demonstration disk for MicroGenie, which runs on IBM-compatible computers. The company is exhibiting in booths 29 and 30.

Pharmacia LKB, exhibiting in booths 88 and 89, has just launched its **PROSIS protein analysis software** package for personal computers (*Reader Service No. 104*). The software can perform secondary structure predictions, based upon the Chou-Fasman and Robson theories; calculate the maximum amino acid homology



The screen display of Pharmacia LKB's DNASIS.

between two sequences; perform molecular weight and homology searches; plot homologies; and perform hydrophobicity analyses. PROSIS has keyword search and database access functions, and can be used with an optional CD-ROM disk that contains both the GenBank and National Biomedical Research Foundation databases. Version 3.0 of Pharmacia-LKB's DNASIS DNA analysis software has also recently been released. DNASIS has been updated to perform homology searches in one-third of the time required by previous versions, and to incorporate improved colour graphics. The new version also allows the automatic entry of fragment migration distances from an optional sonic digitizer for the creation of restriction

maps in either circular or linear formats.

IntelliGenetics last year released version 3.0 of its KeyBank data bank of **protein and nucleic acid sequence patterns** for use with the company's suite of software for mainframe computers (*Reader Service No. 105*). KeyBank consists of over 1,200 functional and structural sequence patterns — referred to as "keys" — derived from protein and nucleic acid sequences published in the scientific literature. IntelliGenetics says KeyBank gives researchers a more efficient way to examine the burgeoning nucleic acid and protein sequence databases. KeyBank contains 16 categories of protein keys, including amino acid modifications, signal sites, binding sites, and enzyme and allosteric sites, and 112 categories of nucleic acid keys, including DNA structure, regulatory regions, binding sites, repeats and ends. IntelliGenetics will have information on KeyBank in booth 27.

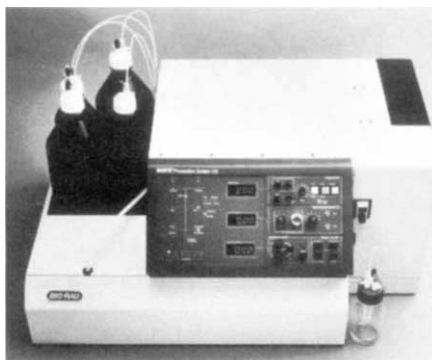
DelPhi is the name of a soon-to-be-released software package from Biosym for calculating the **electrostatic potential** of macromolecules (*Reader Service No. 106*). *DelPhi* enables users to examine the effects of charge distribution, ionic strength, and dielectric constant on the electrostatic potential of a macromolecule without resorting to dielectric models or including explicit solvent. There is no limit on the number of atoms, residues or molecules that can be treated. Biosym says the software yields results that agree within five per cent with analytic solutions for simple spherical models. The output electrostatic potential grids may be viewed as 3-dimensional contour maps using Biosym's *Insight* modelling software. *DelPhi* also generates a dielectric map identifying points inside and outside a molecule's solvent-accessible surface, and a list of potentials at selected coordinates and total electrostatic energy. Biosym is in booth 87.

Pure protein (and DNA)

J.T. Baker, in booth 69, has a new **non-porous polymeric matrix** named Natrix for the purification and concentration of DNA and RNA (*Reader Service No. 107*). Separation with Natrix is achieved by the selective binding of the nucleic acid to the phase in an ion-pairing buffer. The minimum capacity of the matrix is 50 µg of nucleic acid for 200 mg of Natrix. The nucleic acids are eluted using 60 per cent acetonitrile. Baker says Natrix allows the recovery of normal nucleic acids in quantitative amounts, without impairing their capacity for ligation. According to the company, Natrix can be used to purify ten samples of DNA from a caesium chloride gradient in less than 30 minutes. Natrix is available in prepacked Bakerbond columns or as a bulk sorbent.

Boehringer Mannheim Biochemicals, in booths 81-83, has introduced Resorufin-labelled casein, a non-specific substrate for detecting a wide variety of **proteases** that contaminate various protein preparations (*Reader Service No. 108*). The company says the new substrate is both reliable and sensitive in the determination of protease activity: each lot is tested with sequencing-grade trypsin, and as little as 0.1 µg trypsin can produce an absorbance difference of 0.07 at 574 nm in 15 minutes at 37 °C.

Bio-Rad recommends its MAPS preparative system for the **process-scale purification of protein** (*Reader Service No. 109*). Bio-Rad says the MAPS can be used in its integral sanitation method for purifying grams of monoclonal antibodies and



The MAPS system from Bio-Rad.

other proteins per day in a completely sanitized environment. The MAPS system can be treated with 1 M sodium hydroxide, and has an IBM-compatible computer for system control, data collection and manipulation, methods development, and automated sample injection. Compatible column chemistries include Bio-Rad's Affi-Prep protein A columns for MAb's, HRLC MA7 ion-exchange columns for high-molecular weight proteins, macroporous ABCM cation exchange columns for concentration from tissue culture media, and Affi-Prep polymyxin supports for removal of endotoxins. Bio-Rad is exhibiting in booth 3.

Cloning and sequencing

Promega, in booth 54, offers its TaqTrack sequencing system — based on the thermostable DNA polymerase from *Thermus aquaticus* — for sequencing applications (*Reader Service No. 110*). Promega says as a sequencing enzyme, *Taq* exhibits consistent band intensity, low background, and a high degree of accuracy. Because *Taq* works at 70-95 °C, the TaqTrack system decreases the secondary structure of DNA templates, permitting polymerization through highly structured regions. The system is optimized to produce readable sequence data from 1 to 500 bases with a single-stranded DNA template. A deaza nucleotide mix which substitutes 7-deaza dGTP for GTP is avail-

able for resolving band compressions associated with GC-rich templates.

BRL has two **cDNA cloning systems** for cloning double-stranded cDNA into the bacteriophage vectors λgt10 and λgt11 in as little as three days (*Reader Service No. 111*). Based on *EcoRI* linker addition, both cloning systems guide the researcher through all of the cDNA cloning steps: methylating internal *EcoRI* sites, repairing DNA termini, phosphorylating linkers, ligating linkers to DNA, digesting linkers, removing linkers by chromatography, and ligating DNA to the vector. Each step is outlined in the manual, which also includes troubleshooting instructions. The systems include positive control DNA to monitor the success of each step which makes phage with insert DNA form blue plaques and phage containing linkers remain colourless. Each system contains reagents for performing five reactions, with 0.5–2.5 µg cDNA per reaction. BRL is in booths 76 and 77.

The Base Runner **multi-gel electrophoresis sequencer** from International Biotechnologies, Inc. accommodates the independent running of two 32-lane gels simultaneously (*Reader Service No. 112*). The Base Runner holds two 20.3 cm-wide gels of either 30, 45 or 60 cm in length. It has a lockable base which can be rotated 360°, and a buffer tank clamping system that IBI says eliminates user error and plate breakage while reducing setup time. The spring-loaded clamp allows users to slide tanks into place: releasing the clamp causes the tank's silicon gasket to compress against the glass plate, forming a leakproof seal. The Base Runner's gel sandwich is composed of a specially treated notched glass plate and a glass plate coated with a thermoconductive material that IBI says prevents "smiling". IBI is exhibiting in booth 43. □

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Reader Service No.45

Turning the switch on skills

Richard Pearson

Industrial concerns may have to rethink their in-house training programmes — or pay dearly for the skilled workers they need.

WITH the dramatic downturn in the numbers of school-leavers available to enter the labour market and higher education systems of most Western countries, (*Nature* 355, 100; 1988), attention is turning to the existing workforce: after all, 70 per cent of those who will be in employment at the end of the century are already in the workforce.

In response to the growing shortage of technical staff, a number of companies are now embarking on special initiatives to retrain non-technical staff and are widening their recruitment net to embrace new types of trainees by setting up conversion courses. Others are introducing special career streams for technical staff to improve their long-term career prospects, and hence retention rates (*Switching on Skills*, NEDD, 1989).

An example of one of these initiatives can be found at the BBC (British Broadcasting Corporation) which has a continuing need to recruit engineering staff to maintain its broadcasting and transmission activities. In the early 1980s the corporation started experiencing increasing difficulty, along with most other employers, in attracting sufficient engineering graduates and school-leavers qualified in mathematics and physics to enter its training schemes. It foresaw these difficulties worsening, and so decided to widen and adapt its graduate training scheme to include arts graduates. When the places were advertised there was a massive response with 8,000 enquiries, and 3,000 applications for the 24 places. An 'O'-level in maths at grade B, or better, was deemed to be the minimum technical knowledge needed and special training tests were used to aid the selection process.

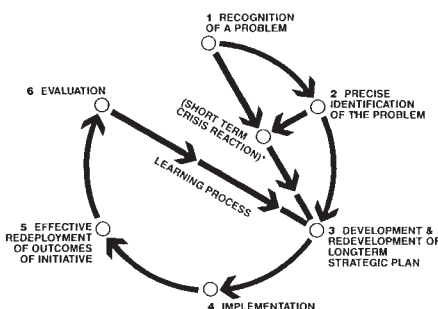
In this way candidates were assessed in near normal training conditions to evaluate their suitability and probable success in the longer term. A special programme was developed to give the trainees the basics of physics in a three-week module. They then followed the 'A'-level trainee programme, both streams eventually merging with the engineering graduate group. The initial off-the-job part of the programme lasts about 13 weeks, and is followed by an 18-month period embracing both off- and on-the-job training.

Although still in its early stages, the programme has been deemed a success, with none of the trainees failing. The BBC has found a completely new source of

recruits, reducing their need to compete in the market for experienced engineers. It is argued that as overall salary levels do not have to match the top of the market, the training costs involved, which exceed £10,000 per trainee for the initial 13 weeks alone, are justified.

Another approach to widening the recruitment net and using non-technical recruits has been undertaken by part of the electronics group Plessey. A shortage of real-time programmers was the spur here, and the company looked to retain internal staff. An in-house 26-week course was designed which could not only take students from a wider range of backgrounds than the external equivalent but was also 60 per cent cheaper. It includes foundation elements of maths and electronics as well as the basics of software. This is followed by short modules on specialist high-level languages and project work. Staff and ex-students have described it as compressing two years of university study into six months.

Initially the course was aimed at graduates but more recently a base entry standard of good 'O'-levels was accepted



The best of training programmes could fail if it is not adapted in the light of experience.

for staff who might otherwise have been in danger of redundancy elsewhere in the company. The course was externally validated and in the second year the Manpower Services Commission, a government agency, arranged to buy places to retain graduates and to help ease the more general skill shortages. Selection was by aptitude test and interview with a notional target age range of 20–30 years, reflecting the perceived market prejudice against older workers. All 90 of the students taking part so far have successfully completed the course; the benefits of 'growing your own' having been emphasized by high retention rates. For the externally sponsored students, normally graduates

with no information technology experience and from backgrounds ranging from civil engineering to the arts having been in employment for up to 5 years, the courses were seen to be highly practical. Their relatively short duration, when compared with a 12-month MSc, means that students can find financial support more easily. By allowing externally sponsored students to participate, the company has been able to continue with the course even in times of slack internal demand.

At Barr and Stroud, manufacturers of electro-optical systems, the concern was to provide career routes for specialist staff and improve retention by allowing career progression outside the traditional management hierarchy. The company has introduced a number of new programmes including a career structure with three streams for professional, specialist and managerial staff, explicit grade and progression criteria, an integrated and visible salary structure, and an appraisal system. The latter is a critical means of identifying training needs and succession planning. The programme, which is currently being implemented, will, it is hoped, result in clearer and better career prospects for staff, leading to lower staff turnover.

For other companies interested in developing such initiatives there are a number of lessons of value from these and the complementary case studies (presented in *Switching on Skills*). The first is the need is to recognize the nature and durability of the problem, such as recruitment difficulties, high wastage or poor utilization. Often this is done but only leads to short term, or crisis reactions. The real need is to identify the precise problem and develop a strategic response. Implementation of the plan then follows; it is at this stage that many initiatives stop. In many cases, however, staff are retrained and then their new skills are not exploited. The programme needs to be evaluated and the lessons fed back into the design and implementation strategies (see figure). Few programmes are 100 per cent successful the first time, and even where they are the problem or the environment will continue to change. The challenge now is to increase the skills of the existing workforce to complement the new skills provided by the young. □

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